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The USAMRDC has developed a "blood substitute" consisting of a cross-linked human hemoglobin in saline solution. The rationale for developing such a product is that it might be useful in the acute resuscitation of soldiers in the battlefield, and also might have utility as an oxygen-carrying volume expander in situations where administration of blood is logistically difficult. In animal models, this material can sustain life in the absence of red cells. It is also effective in resuscitating experimental animals after acute hemorrhage. A common side effect of XL-Hgb administration in animals has been marked arterial and pulmonary hypertension. While the mechanism of this hypertension is unknown, it is thought that XL-Hgb scavenges the endogenous vasodilating substance nitric oxide (NO). In the execution of this contract our group has attempted to determine the effects of this XL-Hgb administration on 1) NO-mediated vasodilation in isolated blood vessels; 2) the metabolism of vasoconstricting catecholamines in the adrenal medulla and sympathetic nerve endings; 3) NO-mediated vasodilation in vivo; and 4) the effects on renal sodium and volume regulation in vivo. The studies conducted as part of this contract generally confirm the hypothesis that XL-Hgb interferes with NO function on isolated tissues in whole animals. It also appears that other vasoconstricting mechanisms might contribute to the hypertension observed during XL-Hgb administration.

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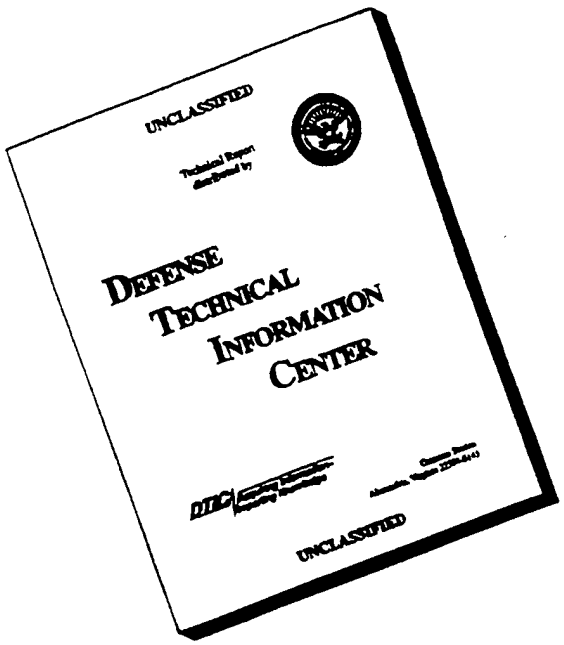

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INTRODUCTION

Overview

It is well recognized that a major threat to injured combat soldiers and other trauma victims is exsanguination (16-18). In some of these patients timely replacement of blood loss with an oxygen transporting volume-expanding solution could be life saving. Additionally, there are a variety of logistical and other problems related to the collection, storage and administration of red blood cells, and in some military environments ensuring an adequate supply of blood in close proximity to where casualties occur might be difficult (5). Additionally, in the last fifteen years, there have been increased concerns related to the transmission of infectious disease via blood transfusions. In an effort to address these and other issues, a variety of candidate "blood substitutes" containing modified hemoglobin compounds are currently in the process of development (5,17,18). The United States Army has developed a cross-linked hemoglobin (XL-Hgb) solution that has been effective in sustaining life in animal models in the near-absence of red blood cells (4,7-9,15). However, a number of problems have been identified with this compound. These problems include a relatively short physiologic half-life, and the observation of systemic and pulmonary hypertension in many animal models (5-9). At the beginning of this contract the concept was that XL-Hgb scavenges endogenous vasodilator nitric oxide (NO), thereby limiting its ability to relax blood vessels (1,5,9,10,12-14). This concept, was generally confirmed in the current series of studies, and is also consistent with emerging evidence related to the "transport" of NO-related compounds by hemoglobin and red blood cells (11). Finally, it appears that non-NO mediated mechanism might contribute to the hypertension caused by XL-Hgb. With this information as a background, our original technical objectives included:

Objective 1: To determine if XL-Hgb transfusion inhibits the production and function of endothelium-derived NO.

Objective 2: To determine if the local vasoconstricting XL-Hgb solutions might be reinforced by increased release of catecholamines and vasoconstricting factors from the adrenal gland and postganglionic sympathetic nerves.

Objective 3: To determine if XL-Hgb disturbs local endothelial and sympathetic control of vascular tone in vivo and if baroreflex control of peripheral vascular resistance is altered.

Objective 4: To determine if hypertension after XL-Hgb transfusion is maintained in spite of "normal" renal hemodynamics due to a disruption of the NO-mediated pressure-induced natriuresis usually seen during volume expansion in hypertension.

In general these objectives were addressed, and when warranted additional related issues were also addressed. However, due to budgetary constraints and the early termination of this contract, some of the originally planned studies were modified or not performed.

Previous Work

There have been attempts to develop "blood substitutes" since the last century. Since blood performs a host of physiologic functions in addition to the transport of oxygen and maintenance of intravascular blood volume, a more appropriate name for "blood substitutes" would be "oxygen-carrying volume-expanding solution." However, the term "blood substitute" is the widely accepted parlance (4,17,18). Beginning in the 1930s there were attempts to make hemoglobin-containing blood substitutes. Along these lines, there have been numerous demonstrations that animals could survive for up to several days in the absence of native red blood cells in the presence of hemoglobin-containing blood substitutes (2,3,5,7,17,18). Additionally, there have also been a variety of demonstrations (in animals) suggesting that these compounds would be effective in the fluid resuscitation of individuals suffering from hypovolemic hemorrhagic shock. There have been several persistent problems shown with all hemoglobin-based blood substitutes: 1) limited intravascular half-life 2) hypertension; 3) potential for long-term disruption of renal function; 4) development of a "pure" hemoglobin solution free of red blood cell stromal debris. Over the years, issues 1 and 2 have been addressed using several techniques. The intravascular half-life of these compounds has been prolonged by chemical cross-linking so that the hemoglobin (once liberated from the red blood cells) remains in a tetrameric unit and does not break down into dimers and monomers (4,7-9,15). The potential for renal damage is also reduced via the cross-linking process. Additionally, better purification of the hemoglobin and removal of red cell stromal debris from these products has markedly reduced potential renal complication (issues 2 and 4). In this context, one of the most important of the newer generation hemoglobin solutions currently under development by a variety of governmental and industrial concerns is the U.S. Army Research and Development Command's alpha-alpha 9 XL-Hgb (4,5,7-9,15,17,18). Several important features of this molecule deserve attention:

1) It continues to represent the gold standard for purified hemoglobin.

2) Non-infectious material can be prepared from red blood cells infected with various viral contaminants including HIV.

3) A variety of transfusion protocols in animal models suggests that this molecule is effective in restoring blood volume in animals subjected to experimental hypovolemic hemorrhagic shock. Additionally, animals can survive for prolonged periods of time after complete exchange transfusions with cross-linked hemoglobin solutions.

4) The major continuing "toxicity" of cross-linked hemoglobin solutions given to animals appears to be systemic and pulmonary hypertension as a result of vasoconstriction.

Purpose of the Completed Work

With this information as a background, the purpose of the work outlined in this contract was to better understand the mechanisms responsible for the hypertensive effects of XL-Hgb when given to animals. In project 1 Dr. Katusic used classic pharmacologic techniques on isolated blood vessels to "pharmacodissect" the interactions of XL-Hgb and the nitric oxide pathway that regulate vascular tone in a variety of blood vessels. In project 2 Drs. Rorie and Tyce studied the concept that nitric oxide is a key regulator of catecholamine release from both the adrenal medulla and sympathetic nerves. In project 3 Dr. Joyner studied the effects of XL-Hgb on baseline vascular tone in a variety of organ systems and on the vasodilator responses to a variety of pharmacologic compounds. The planned studies on the effects of XL-Hgb on baroreceptor regulation of blood pressure were abandoned due to budgetary cuts. In project 4 (Dr. Romero) the effects of XL-Hgb on renal blood pressure regulating mechanisms were being evaluated. The key finding in these studies is that when XL-Hgb was given as a volume expander, it is one of the only solutions that causes both volume expansion and hypertension without evoking natriuresis and diuresis. This is consistent with the concept that NO is a key mediator of these responses.

Methods and Approach

Overview

Four laboratories collaborated on interrelated projects to address the mechanisms of hypertension after XL-Hgb blood substitute transfusion.

1) *Dr. Katusic.* In Dr. Katusic's laboratory, standard *in vitro* pharmacologic techniques in isolated blood vessels were used to explore the pharmacologic effects of XL-Hgb on the normal contracting and relaxing factors which regulate blood vessel tone.

2) In Project 2 *Drs. Rorie and Tyce* investigated the interactions of XL-Hgb, nitric oxide, and catecholamine release from the adrenal medulla and sympathetic nerves. Their laboratory is fully equipped and capable of making these measurements.

3) In Project 3 *Dr. Joyner* investigated the effects of XL-Hgb administration on *in vivo* pharmacologic responses related to the NO pathway in an anesthetized canine model.

4) In Project 4 *Dr. Romero* investigated the effects of volume expansion with XL-Hgb on renal blood pressure regulating mechanisms.

Techniques

Animal Model. Studies were conducted in anesthetized dogs and isolated canine blood vessels and adrenal glands. Animals were housed and cared for in accordance with AAALAC standards and Institutional Animal Care and Use Committee guidelines. All studies were approved by the Institutional Animal Care and Use Committee. The total number of animals used was reduced since investigators that study isolated blood vessels obtained these tissues from a common shared source animal that services the needs of multiple laboratories. This approach provides the maximum yield of tissue using the minimal number of animals. Additionally, the budgetary cutbacks during the final year of the study reduced the number of animals used to address issues associated with objectives 3 and 4.

Animal Instrumentation (general). Dogs were anesthetized with pentobarbital (30 mg/kg, maintained with supplemental doses of 10 mg/kg). They were subsequently intubated and mechanically ventilated to maintain normal arterial blood gases. Body temperature was maintained at 36-38°C. Arterial pressure was monitored with an indwelling catheter located in a femoral artery. Central venous pressure (CVP) and cardiac output (some studies) were measured using thermodilution with a pulmonary artery catheter advanced from the femoral or jugular vein. Regional (mesenteric, iliac, renal, or coronary) arterial blood flows were measured using *Transonic* flow probes placed around the vessels of interest after laparotomy or sternotomy. Arterial and central venous pressure, along with regional blood flows, were measured continuously. Arterial blood gases and cardiac output were measured every 10-15 minutes. In some studies, small polyethylene cannulas were placed into the proximal portions of the arteries of interest for local infusion of drugs. After physiological measures were completed, the anesthetized animals were sacrificed using potassium chloride, barbiturate overdose, or exsanguination. Further technical details are available in the appended manuscripts. Specific details of the large animal instrumentation are available in the materials submitted that are relevant to projects 3 and 4.

XL-Hgb Transfusion. XL-Hgb from the USAMRDC has been used in two basic transfusion protocols. Hypovolemic partial exchange transfusions were conducted after removal of 30-40 cc/kg of blood or sufficient blood volume to cause an ~30% reduction in mean arterial pressure (MAP). This was followed immediately by resuscitation with 60 cc bolus doses of XL-Hgb sufficient to return MAP to baseline. Sham control experiments were also conducted using the approaches outlined above, but the volume replacement consisted of commercially available albumin or dextran solutions designed to have a viscosity and osmolality similar to that of the XL-Hgb product. Volume expansion was also used to expand the blood of a normovolemic animal by 10-20% with either XL-Hgb, albumin, or dextran. Total hemoglobin is estimated measuring the hematocrit and the Hgb concentration in spun plasma. These approaches were used in projects 3 and 4.

Drugs. A variety of compounds were used. Details of the specific experimental approaches are available in the appended abstracts and manuscripts submitted with the materials relevant to each project.

Chemical Determinations:

Catecholamines in the presence of XL-Hgb. Over the past 13 years, the laboratory of Drs. Tyce and Rorie has developed many of the standard catecholamine assays used internationally which yield recoveries of >80% of added authentic standards. However, it was impossible to recover catecholamines from fluids containing XL-Hgb using the traditional methods. In this context, Drs. Rorie and Tyce developed new or modified their standard techniques and collaborated with the Mayo Mass Spectrometry Core to identify these compounds. Further details associated with these issues are contained in the project 2 report.

Isolated Blood Vessel Preparations (Projects 1 and 2):

Tensions developed in blood vessels. Studies have been conducted in organ baths on isolated canine mesenteric, renal, left circumflex coronary, femoral, and other large arteries. Blood vessels with and without endothelium were prepared using the standard techniques that remove endothelial cells, but preserve the ability of the vascular smooth muscle to contract. The presence or absence of endothelium was confirmed by determining whether or not bradykinin causes relaxation during contractions evoked by an EC_{50} concentration of contractile agonists. All experiments were performed in the presence of indomethacin in order to inhibit activity of cyclooxygenase. Further details are presented in the materials related to project 1.

NE release from sympathetic nerve endings in vessels. The details of the approaches used by Drs. Rorie and Tyce to study these issues are outlined in their appended abstracts and manuscripts. Further details are presented in the materials related to project 2.

PROJECT 1

"Effects of XL-Hgb on Endothelial Regulation of Vascular Tone"

Dr. Z.S. Katusic

This project was designed to determine the effect of XL-Hgb on the function of endothelial and smooth muscle cells of isolated peripheral and cerebral arteries. Our major goal was to determine the effect of XL-Hgb on endothelium-dependent relaxations mediated by nitric oxide. Previous studies from a number of different laboratories, including our own, reported that hemoglobin is a potent chemical antagonist of nitric oxide (1). These results suggested that the most likely mechanism responsible for the pressor effect of XL-Hgb is chemical inactivation of nitric oxide. Furthermore, previous studies on isolated cerebral arteries have also demonstrated that hemoglobin is a potent vasoconstrictor and that this effect is not dependent on the presence of endothelial cells (1). More importantly, the effects of hemoglobin have not been systematically studied on isolated small resistance arteries that play a key role in regulation of arterial blood pressure. Thus, initially we focused our attention on the effects on XL-Hgb in large arteries and than analyzed the effects of XL-Hgb in small resistance arteries.

Our studies were performed on isolated canine femoral, renal, coronary and basilar arteries. Standard organ chamber technique was used to study vascular reactivity. We also used radioimmunoassay to measure production of cyclic GMP and cyclic AMP in vascular wall in order to determine the effects of XL-Hgb on the levels of major vasodilator second messengers in smooth muscle cells. Detailed description of these techniques is provided in enclosed copy of the published manuscript.

To study reactivity of small resistance arteries treated with XL-Hgb, secondary branches of basilar arteries and tertiary branches of mesenteric arteries (inner diameter ~ 300 μm) were dissected under dissection microscope. The arteries were than transferred to an arteriograph filled with oxygenated (95% O_2 and 6% CO_2) control solution and than mounted onto microcannulas (Living System Instrumentation, Burlington, Vermont). Control solution circulated from a 250 ml oxygenated reservoir through the arteriograph chamber at flow of 12 ml/min. Temperature was continuously monitored to maintain the vessel environment at 37±0.5°C. The arteriograph was placed on the stage of an inverted microscope (Diaphot-TMD, Nikon) that had a video camera attached to the viewing tube. The signal derived from the video image of the vessel was processed by an electronic system (Living System Instrumentation) for the continuous measurement and recording of both inner diameter and wall thickness.

Responses of the pressurized arteries (in the absence of intraluminal flow) were measured at a transmural pressure of 50 mm Hg. This pressure was found to be optimal for contractions of these arteries as assessed by repeated exposures to $3x10^{-8}$M U46419 (a thromboxane A_2 receptor agonist) at various transmural pressures.

Effects of XL-Hgb in large arteries

The experiments on large arteries provided several novel observations that may help to explain cardiovascular effects of XL-Hgb. It is clear from our experiments that in the presence of relatively low concentrations of XL-Hgb (10^{-7} and 10^{-6}M), production of nitric oxide in endothelial cells is impaired. Measurements of cyclic nucleotides demonstrated that XL-Hgb selectively abolished formation of cyclic GMP under basal conditions, or during activation of the endothelium with acetylcholine. The selectivity of the effect of XL-Hgb was demonstrated by the fact that it did not affect production of cyclic AMP. These observations are best explained by chemical antagonism between nitric oxide and hemoglobin. More importantly, the inhibitory effect of XL-Hgb on endothelium-dependent relaxations mediated by nitric oxide was detected in peripheral and cerebral arteries. This observation is consistent with results of previous studies demonstrating that oxyhemoglobin is a potent inhibitor of endothelium-dependent relaxations (1,2). Indeed, the results of this project demonstrated that chemical modification of hemoglobin molecule by crosslinking with bis(3,5-dibromosalicyl) fumarate does not affect its ability to inhibit relaxations mediated by increased production of nitric oxide (3). Thus, our studies clearly demonstrated that XL-Hgb impairs endothelium-dependent relaxations in isolated large conduit arteries. Decreased availability of nitric oxide in the presence of XL-Hgb is reflected in selective inhibition of cyclic GMP formation. This in turn may help to explain pressor effect of XL-Hgb. Furthermore, under in vivo conditions impaired function of nitric oxide may increase aggregation platelets, adhesion of white blood cells and proliferation of smooth muscle cells.

Effects of XL-Hgb in small resistance arteries

It is generally believed that the role of nitric oxide in control of vascular tone in resistance arteries is less prominent than in large arteries. On the other hand it has been proposed that in small arteries nitric oxide may act more efficiently as an inhibitor of leukocyte adhesion and platelet aggregation as well as modulator of vascular growth (4). To examine the reactivity of small vessels to nitric oxide, we examined the effect of a nitric oxide donor, 3-morpholinosydnonimine (SIN-1) in secondary branches of basilar arteries contracted with endothelin-1 (10^{-9}-10^{-8}M). SIN-1 (10^{-8}-10^{-4}M) caused concentration-dependent relaxations. This effect of SIN-1 was almost abolished in the presence of XL-Hgb (10^{-5}M; Figure 1), suggesting that nitric oxide is a potent vasodilator in these arteries, and that XL-Hgb is an inhibitor of relaxations to exogenous nitric oxide. Because our previous studies suggested that in resistance arteries a portion of endothelium-dependent relaxations to bradykinin and calcium ionophore A23187 (5) may be independent of nitric oxide production, we examined the effect of XL-Hgb on these relaxations. Interestingly, despite the fact that we used very high concentration of Xl-Hgb endothelium-dependent relaxations to bradykinin were not abolished (Figure 2). In addition, XL-Hgb caused modest reduction of endothelium-dependent relaxations to A23187 (Figure 3). These results suggest that in resistance arteries of the brain, endothelial function is impaired by XL-Hgb, however, this impairment appears to be less pronounced than in large arteries, suggesting that nitric oxide may not be a sole mediator of endothelium-dependent relaxations in these arteries. Alternatively, high local concentration of nitric oxide in these arteries may overcome chemical antagonism of XL-Hgb. Selectivity of XL-Hgb inhibitory effect on endothelium-dependent relaxations was demonstrated by the fact that XL-Hgb did not affect relaxations to 8-bromo cyclic GMP (Figure 4).

In contrast to cerebral arteries, in tertiary branches of mesenteric arteries endothelium-dependent relaxations to bradykinin were abolished (Figure 5). At the present time we do not have an explanation for the differential effect of XL-Hgb in cerebral and peripheral arteries. Further studies are needed to fully characterize the mechanisms of endothelium-dependent relaxations in these vascular beds. Based on these findings it will be possible to explain heterogeneous reactivity to XL-Hgb.

In small cerebral arteries we have also examined the effect of Xl-Hgb on relaxations to a ATP-sensitive potassium channel activator pinacidil. The rationale for these experiments was based on the reports in the literature demonstrating an important role of potassium channels activation in control of vascular tone (6). During contractions to endothelin-1, pinacidil (10^{-7}-10^{-4}M) caused concentration-dependent relaxations (Figure 6). This effect of pinacidil was significantly reduced in the presence of XL-Hgb (10^{-5}M). In contrast, XL-Hgb did not affect relaxations to a calcium channel blocker diltiazem (Figure 7). Thus, it appears that XL-Hgb may affect vascular tone not only by inactivation of nitric oxide but also by interacting with potassium channels. The precise mechanisms responsible for this effect of XL-Hgb remains to be determined, however this intriguing observation illustrated complexity of the vascular effects of XL-Hgb.

Summary

The results of this project clearly demonstrated that XL-Hgb in concentration range from 10^{-7}M to 10^{-5}M is an inhibitor of endothelium-dependent relaxations in large arteries. More importantly, XL-Hgb selectively inactivates nitric oxide produced under basal conditions with subsequent significant decrease in levels of cyclic GMP in vascular wall. These inhibitory effects on a key vasodilator mechanism in vascular wall may help to explain pressor effect of XL-Hgb observed in vivo. Interestingly, inhibitory effect of Xl-Hgb on endothelium-dependent relaxations mediated by nitric oxide in resistance arteries was less pronounced. However, in small arteries we detected an inhibitory effect of XL-Hgb on relaxations to ATP-sensitive potassium channel activator pinacidil, suggesting that in these arteries XL-Hgb may inhibit vasodilator mechanisms that are not dependent on production of nitric oxide. Taken together these results support the idea that pressor effect of XL-Hgb, is in part, mediated by inactivation of vasodilator mechanisms responsible for maintenance of normal vascular tone.

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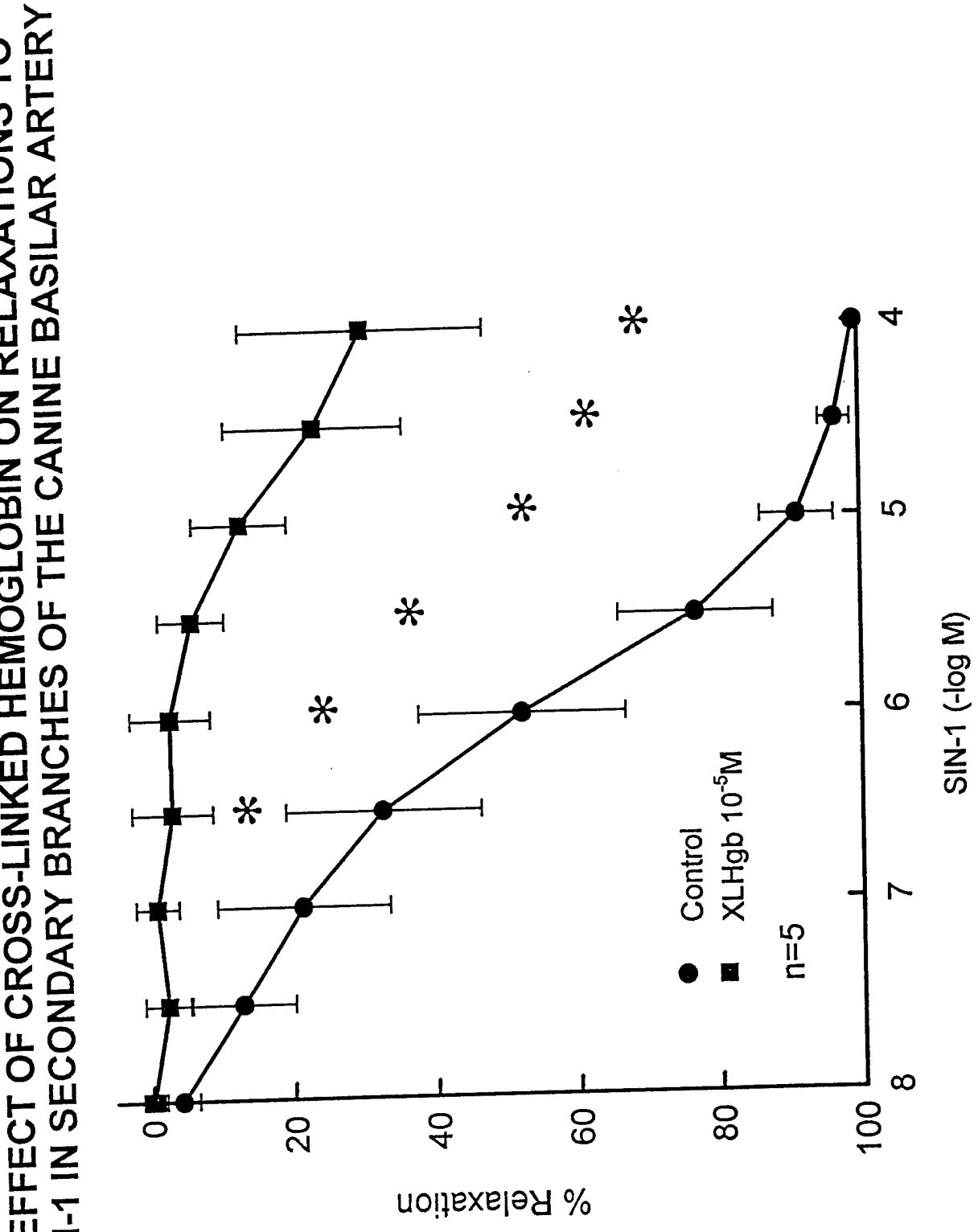
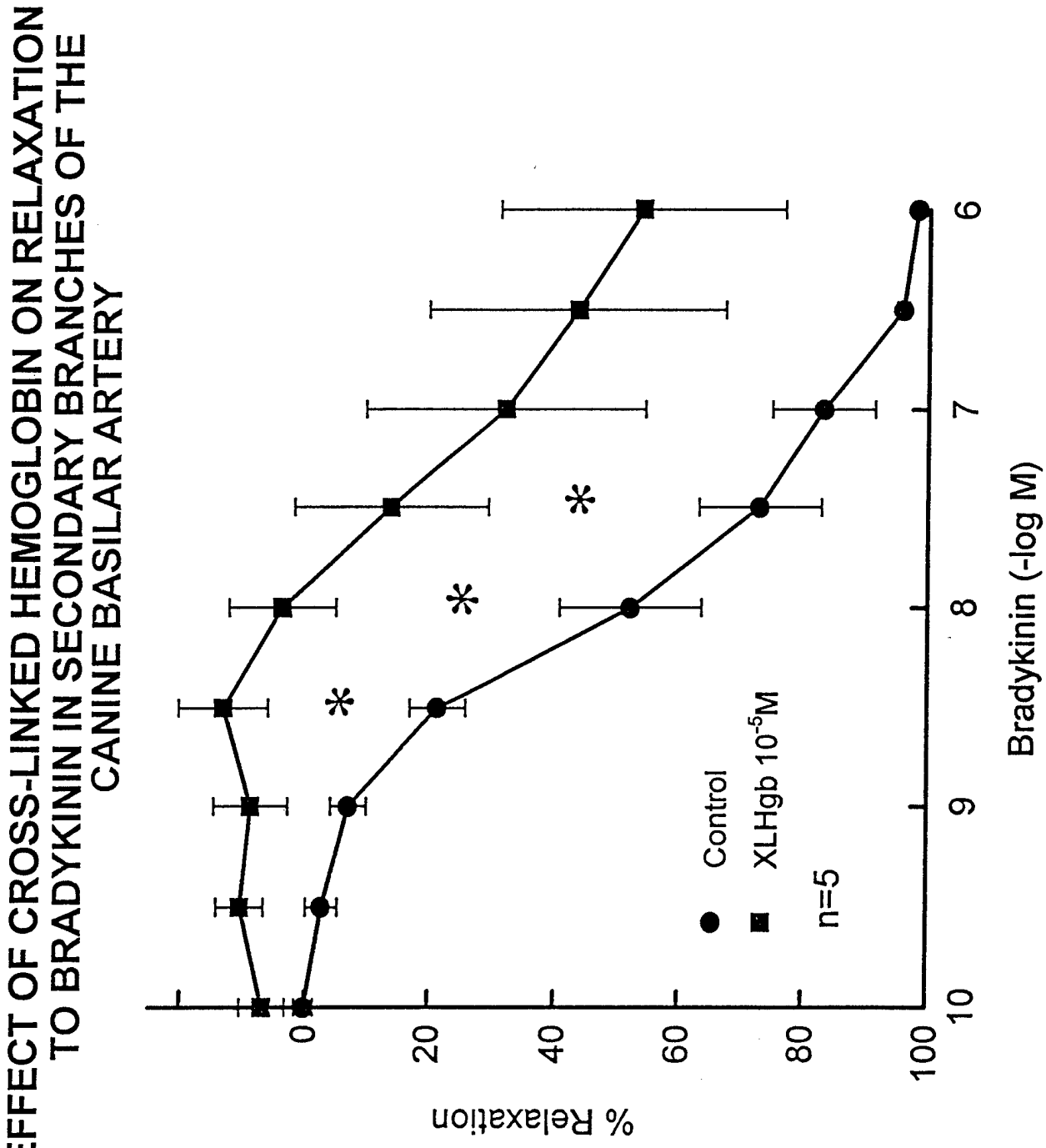
1. Katusic, ZS., Lee, H.C., and Clambey, E.T. Crosslinked hemoglobin inhibits endothelium-dependent relaxations in isolated canine arteries. Gen. Pharmac. 27: 239-244, 1996.

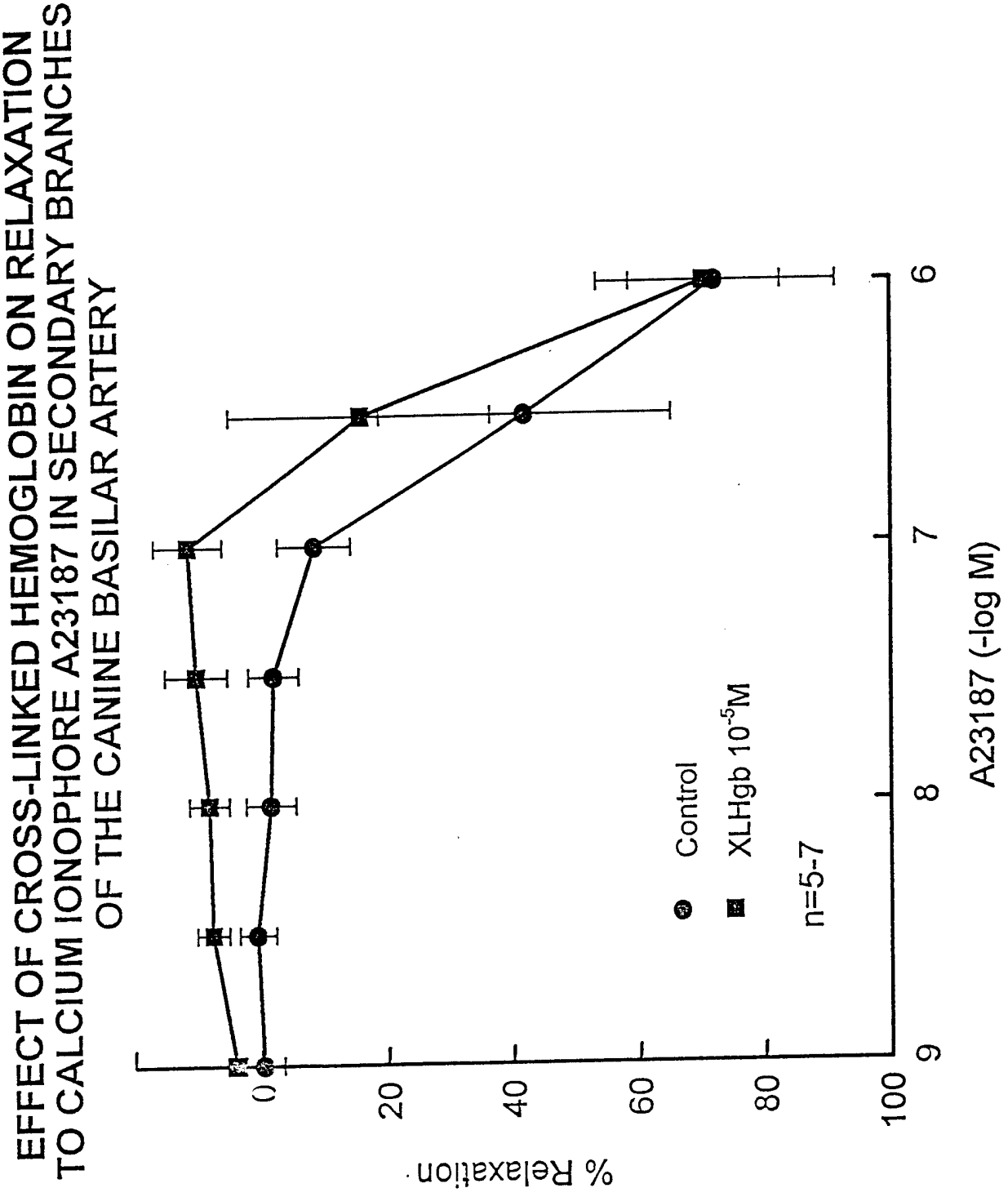
2. Cases, A., Stulak, J.M., Katusic, Z.S., Villa, E., and Romero, J.C. Hemodynamic and renal effects of cross-linked hemoglobin infusion. Am. J. Physiol. (submitted).

3. Katusic, Z.S. Effects of crosslinked hemoglobin on endothelial function in isolated canine resistance arteries. (In preparation)

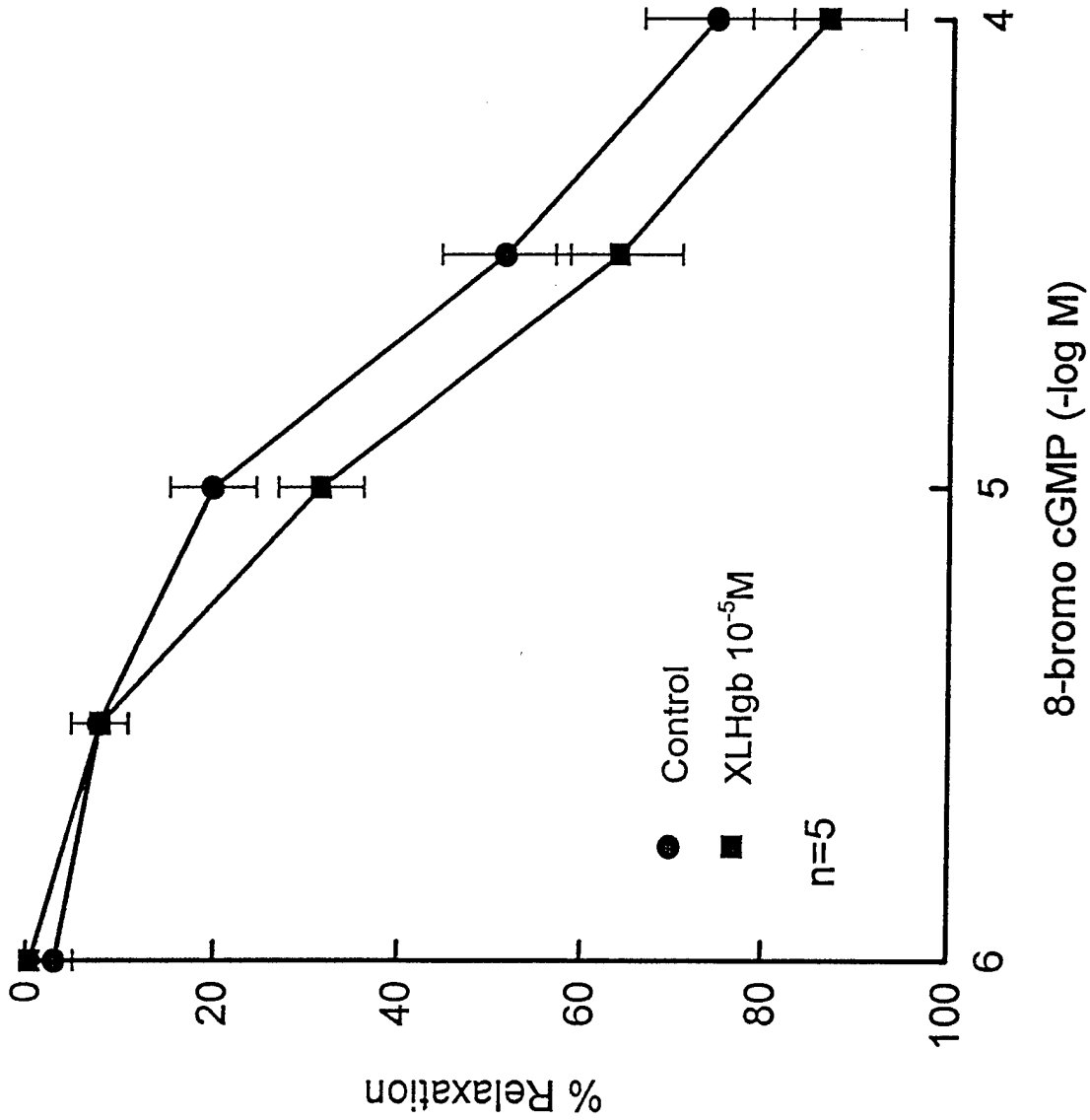
Figure 1

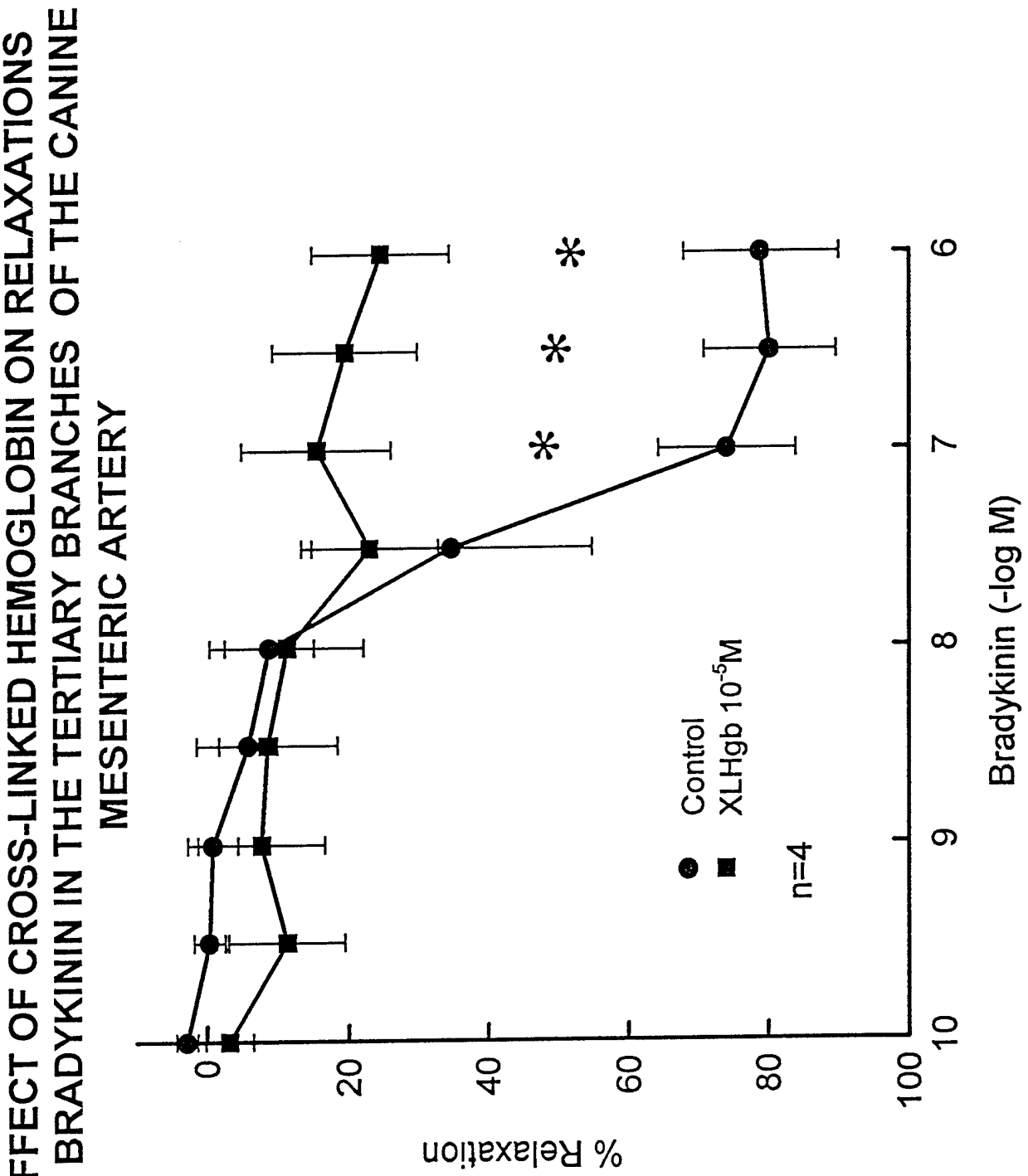

EFFECT OF CROSS-LINKED HEMOGLOBIN ON RELAXATIONS TO SIN-1 IN SECONDARY BRANCHES OF THE CANINE BASILAR ARTERY
% Relaxation
0
20
40
60
80
100
SIN-1 (-log M)
4
5
6
7
8
Control
XLHgb 10-5M
n=5
*
*
*
*
*


EFFECT OF CROSS-LINKED HEMOGLOBIN ON RELAXATION
TO BRADYKININ IN SECONDARY BRANCHES OF THE
CANINE BASILAR ARTERY
% Relaxation
0
20
40
60
80
100
Bradykinin (-log M)
6
7
8
9
10
Control
XLHgb 10-5M
n=5
*
*
*


EFFECT OF CROSS-LINKED HEMOGLOBIN ON RELAXATION
TO CALCIUM IONOPHORE A23187 IN SECONDARY BRANCHES
OF THE CANINE BASILAR ARTERY
% Relaxation
0
20
40
60
80
100
6
7
8
9
A23187 (-log M)
Control
XLHgb 10-5M
n=5-7

EFFECT OF CROSS-LINKED HEMOGLOBIN ON RELAXATIONS TO 8-BROMOGUANOSINE 3':5' CYCLIC MONOPHOSPHATE IN SECONDARY BRANCHES OF THE CANINE BASILAR ARTERY




EFFECT OF CROSS-LINKED HEMOGLOBIN ON RELAXATIONS TO BRADYKININ IN THE TERTIARY BRANCHES OF THE CANINE MESENTERIC ARTERY
% Relaxation
0
20
40
60
80
100
10
9
8
7
6
Bradykinin (-log M)
Control
XLHgb 10^{-5}M
n=4
*
*
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EFFECT OF CROSS-LINKED HEMOGLOBIN ON RELAXATIONS TO PINACIDIL IN SECONDARY BRANCHES OF THE CANINE BASILAR ARTERY

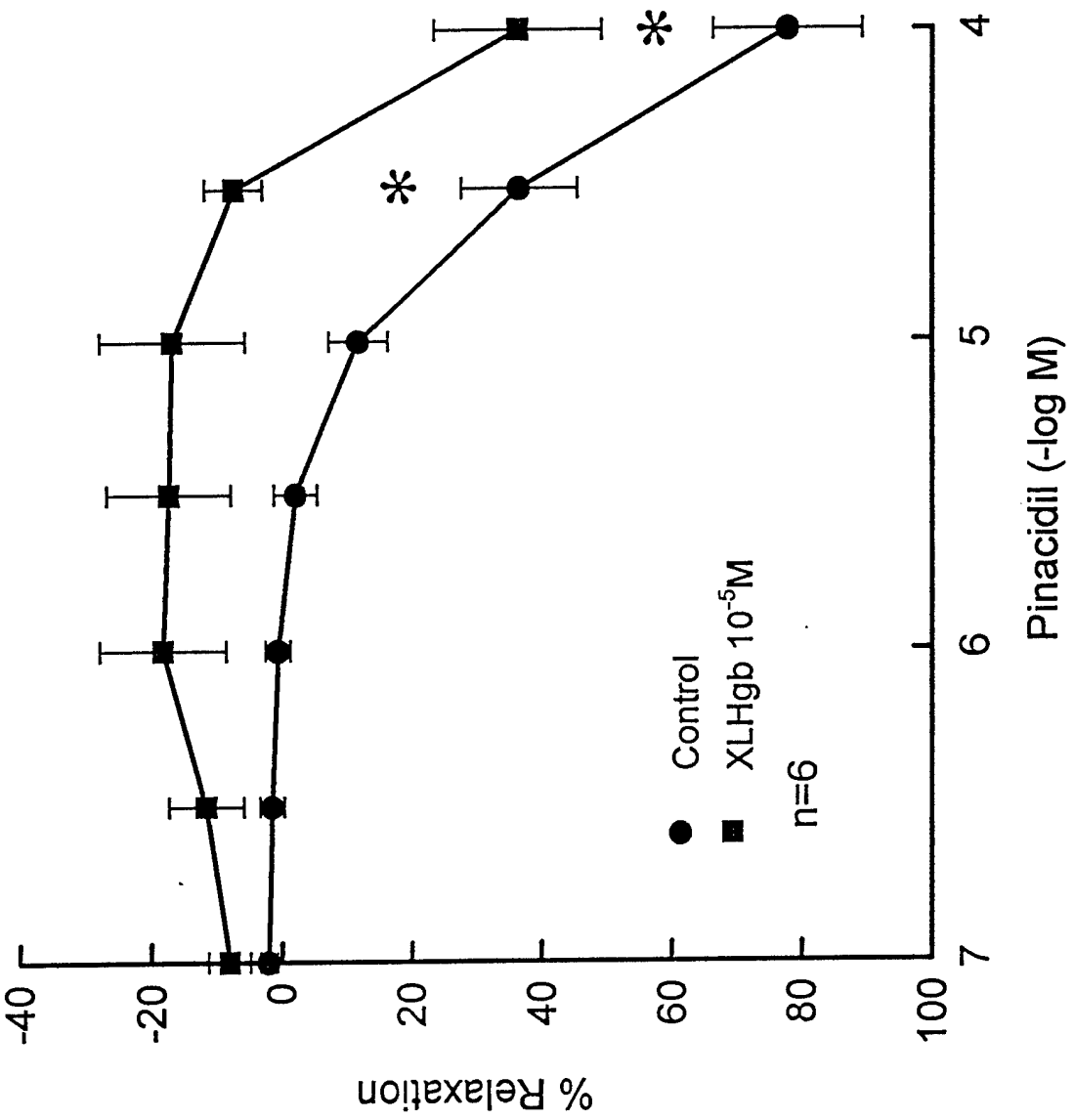
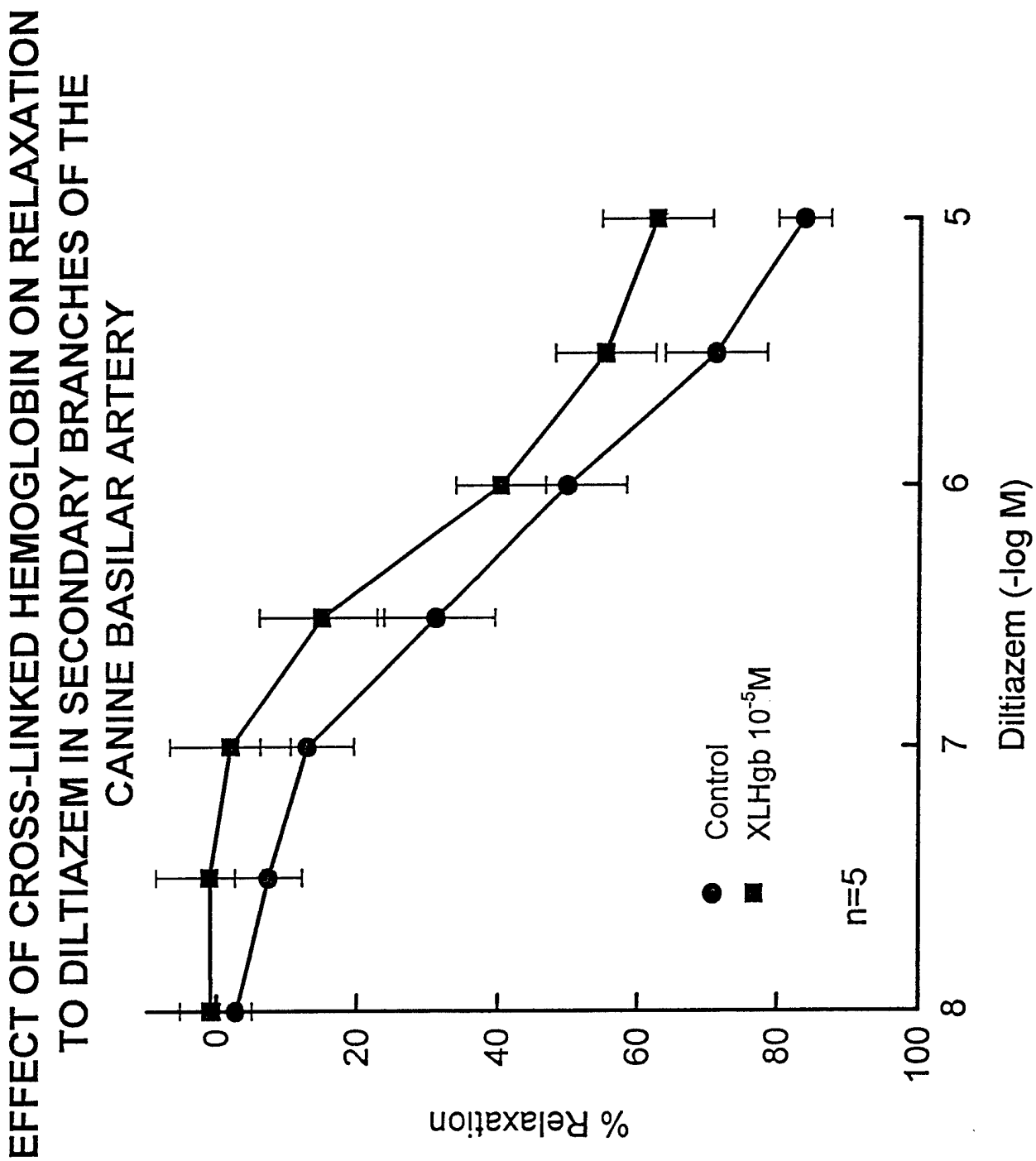


Figure 7


EFFECT OF CROSS-LINKED HEMOGLOBIN ON RELAXATION TO DILTIAZEM IN SECONDARY BRANCHES OF THE CANINE BASILAR ARTERY
% Relaxation
0
20
40
60
80
100
8
7
6
5
Diltiazem (-log M)
Control
XLHgb 10^{-5}M
n=5

PROJECT 2

"Effects of Xl-Hgb Solutions on Catecholamine Release from the Adrenal Medulla and Vascular Sympathetic Nerve Terminals"

Drs. G.M. Tyce and D.K. Rorie

A. Specific Aims of the Project

To determine whether cross-linked hemoglobin (XL-Hgb) affects the release of catecholamines from the adrenal medulla or from sympathetic nerve endings in blood vessels.

B. Publications Supported by Contract #USAMRDC 91283001

Hunter LW, Tyce GM, Rorie DK: Norepinephrine release during vasoconstriction induced by cross-linked hemoglobin. Life Sci, 59:131-140, 1996.

Ward LE, Hunter LW, Grabau CE, Tyce GM, Rorie DK: Nitric oxide reduces basal efflux of catecholamines from perfused dog adrenal glands. J Auton Nerv Syst, in press.

Hunter LW, GM Tyce, Benson LM, Naylor S, Rorie DK: Transmural stimulation of mesenteric artery in the presence of cross-linked hemoglobin produces a compound with chromatographic characteristics similar to dopamine. Biogenic Amines, submitted.

Tyce GM, Ward LE, Hunter LW, Rorie DK: Effects of nitric oxide on overflow of catecholamines from perfused dog adrenal gland. 8th International Symposium on Chromaffin Cell Biology, Edinburgh, Scotland, August 6-10, 1995 (abstract)

Hunter LW, Tyce GM, Rorie DK: Effect of αα cross-linked hemoglobin (XL-Gb) on norepinephrine (NE) release and contraction in femoral artery (FA). FASEB J 10:A341, 1996 (abstract).

Tyce GM, Chritton SL, Barnes RD, Ward LE, Hunter LW, Rorie DK: The adrenal gland as a source of DOPA and of catecholamine metabolites. 8th International Catecholamine Symposium, Pacific Grove, CA, October 13-18, 1996 (abstract).

Barnes RD, Ward LE, Tyce GM, Rorie DK: Role of nitric oxide in modulation of evoked catecholamine efflux from canine adrenal gland. International Anesthesia Research Society 71st Clinical and Scientific Congress, San Francisco, CA, March 14-18, 1997 (abstract).

C. Progress Report

I. Releases from the adrenal glands: Since hemoglobin reacts readily with nitric oxide (NO), it is likely that Hgb-induced effects will be via an interaction with NO. Thus initial experiments were directed towards determining whether NO has a role in the regulation of basal and evoked releases of catecholamines from the adrenal gland.

(i) Basal releases: Effects of endogenous NO. Isolated perfused dog adrenal glands were used in these studies. Basal releases were compared in the presence and absence of N^G-monomethyl-L-arginine (L-NMMA, $3x10^{-4}$M), an inhibitor of nitric oxide synthase (NOS) or of one of a number of NO donors. In the presence of L-NMMA basal effluxes of norepinephrine (NE), epinephrine (E) and dopamine (DA) were increased (Figures 1-3). Those effects were reversed in the presence of L-arginine (10^{-3}M) (Figs 1-3) indicating that endogenous nitric oxide inhibited the basal effluxes of catecholamines from the adrenal gland. Sodium nitroprusside, an NO donor, inhibited the releases of NE and E (see enclosed manuscript).

Basal or spontaneous releases of catecholamines from adrenal gland has previously received scant attention but there are suggestions that this basal efflux is substantial. The importance of this efflux may be the maintenance of sensitivity and proper trophic function of the target organs on which released catecholamines act.

Neither inhibition of NOS nor the addition of NO donors had any effect on basal overflow of the met-enkephalins. The release of these peptides did not appear to be under the influence of NO.

(ii) Basal releases of catecholamines from adrenal gland: Effects of XL-Hgb. XL-Hgb (10^{-4} and 5×10^{-4}M) increased the basal release of E, NE and DA in the isolated perfused adrenal gland. This effect was of a similar magnitude to that of L-NMMA. To determine whether XL-Hgb was exerting its action via an interaction with NO, we studied the effect of 10^{-4}M XL-Hgb in perfusions where NO production is greatly attenuated i.e. in the presence of 3×10^{-4}M L-NMMA. In these circumstances the effects of XL-Hgb were greatly reduced indicating that a major component of the action of L-NMMA is attributable to an interaction with NO. However, the data indicate that a small component of the action of XL-Hgb in increasing basal catecholamine release is independent of an interaction with NO.

These data are currently under analysis and a manuscript is in preparation.

(iii) Evoked releases of catecholamines from adrenal gland: Effect of nitric oxide. Release of catecholamines from adrenal glands of dog, man and most species is evoked by stimulation of nicotinic receptors by acetylcholine. Releases via this mechanism were compared in the presence and absence of L-NMMA using the isolated perfused dog adrenal gland. Releases were evoked with the nicotinic agonist 1,1-dimethyl-4-phenylpiperazinum (DMPP).

Isolated dog adrenal glands were perfused and after 60-min stabilization perfusate was collected during (a) a 10-min basal period, (b) a 2-min stimulation with a "low" ($3x10^{-6}$M) or a "high" ($5x10^{-5}$M) dose of DMPP, a nicotinic agonist, (c) an 8-min post-stimulation, and (d) a 30-

min stabilization period. This stimulation sequence was repeated three times (S_1, S_2, S_3). In some studies L-NMMA was added to the perfusate before the third sequence of collections. E, NE and DA in perfusates were quantified by HPLC. Net evoked catecholamine efflux was calculated by subtracting prior basal efflux from total evoked efflux. The ratios of net evoked catecholamine effluxes in S_3 expressed as a percentage of effluxes in S_2 were compared in the presence vs. the absence of L-NMMA.

Effluxes (as ng/min) of E, NE, and DA during S_2 were: 2050±191, 264±50 and 23±3 with low dose of DMPP, and 4306±664, 1215±197 and 43±3 with high dose of DMPP respectively.

Inhibition of NOS by L-NMMA had differing effects depending on the intensity of stimulation: at low intensity stimulation L-NMMA increased releases, but at high intensity stimulation L-NMMA decreased releases of catecholamines (Table 1).

Table 1. S_3/S_2 Ratios in Presence and Absence of L-NMMA

	Low Dose DMPP		High Dose DMPP	
	Control	with L-NMMA	Control	with L-NMMA
E	80.5±18.8%	112.4±13.3%	73.8±3.1%	58.0±5.7%*
NE	56.0±7.2%	125.7±13.3%*	61.5±2.3%	58.9±5.6%
DA	73.7±5.8%	121.1±19.5%*	82.7±4.6%	63.3±4.5%*

*Significantly different from control (Student's t test).

II. Releases of catecholamines from sympathetic nerve endings:

(i) Effect of XL-HGB or basal and evoked released of NE from nerve endings in blood vessels. Studies were done using isolated superfused segments of canine femoral artery to determine whether XL-Hgb induced increased efflux of NE from sympathetic nerve endings in the vasculature. This blood vessel was chosen because preliminary studies showed that it contracted in response to 10^{-5}M XL-Hgb.

Helical strips of canine femoral artery were prepared and superfused *in vitro* with Krebs-Ringer solution and, for each strip, the overflow of NE into the superfusate as well as contractile responses were measured concurrently during basal conditions, during nerve stimulation and during tyramine-evoked release of NE. XL-Hgb (10^{-5}M) contracted unstimulated strips without affecting NE overflow (Fig. 4). NE overflow also was unchanged by N^G-monomethyl-L-arginine (L-NMMA; 300 μM), an inhibitor of NO synthesis, by sodium nitroprusside (SNP; 1 μM) an NO donor, by a combination of HL-Hgb and L-NMMA, or of XL-Hgb and SNP. These treatments contracted the strips to the same degree as did XL-Hgb alone, except for SNP, which induced relaxation (Fig. 4). Transmural stimulation of the strips at 2 and 10 Hz induced NE overflow and contraction, neither of which was affected by any treatment except SNP which significantly

($P<0.05$) increased NE overflow while inhibiting contraction. In a further series of experiments, XL-Hgb augmented contractions induced by tyramine (10 μM) although the resulting NE release was unaffected. These results suggest that, in the femoral artery, contractions induced by XL-Hgb are not due to increased efflux of NE from vascular nerve endings but are consistent with inhibition by XL-Hgb of the postjunctional actions of NO.

(ii) Release of DA-like compound from XL-Hgb. When sympathetic nerve endings in isolated canine mesenteric arteries were stimulated electrically a compound appearing to be DA was released (Fig. 5 B, C and D). This compound was released in a frequency-dependent manner (Figures 5C and D). Production of this putative dopamine occurred only in arteries exposed to XL-Hgb (compare Figures 5F and 5D). A study was done to determine whether this compound was, in fact, DA. The production of DA, a substance with vasodilatory properties, was of considerable interest primarily because of the heterogeneity in contractile responses to XL-Hgb among different vascular beds.

Several chromatographic characteristics of the unknown compound were identical to those of DA, i.e. adsorption onto Sep-Pak C_{18} cartridges, and isographic elution on a reversed-phase HPLC column. However, the compound did not adsorb onto neutral alumina or onto a cation-exchange resin as did DA, and its voltametric properties were similar but not identical to those of DA. Subsequently, the compound was found to be produced in Krebs-Ringer solution in the absence of artery (Figure 6B), provided XL-Hgb, oxygen and an electric current were supplied (Figure 6, compare H and B). Similar results were obtained when other proteins were substituted for XL-Hgb (Figure 6C, D, E, F, and G). It was concluded that the compound released from mesenteric artery by XL-Hgb was not DA.

In several experiments, we examined the possibility that the unknown compound might influence the release or disposition of NE or might affect contraction in isolated blood vessels. However, no effects on the release or disposition of NE could be demonstrated, and the compound did not cause contractions in segments of dog mesenteric artery. Because of the lack of physiological actions at neuroeffector junctions of the unknown compound, further lines of investigation were not pursued.

Analyses by mass spectrometric procedures in collaboration with Dr. S. Naylor, Director of the Mass Spectrometry Facility at Mayo, indicated that the unknown compound was not DA. However, because of high counts of background ions which interfered with mass spectrometric detection, the compound could not be identified.

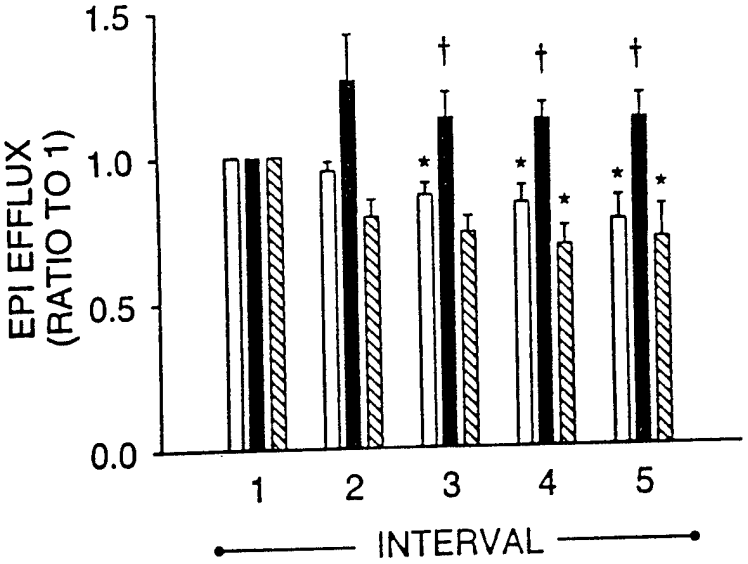


Figure 1. The effect of L-NMMA, 3 x 10^{-4}M, on the basal efflux of EPI from dog adrenal gland. In Figs. 1-3 L-NMMA, when added to the perfusate, was present in intervals 2-5. Ca^{2+} was present in all perfusates. Data are the means ± SEM of 5 to 9 experiments. Three L-NMMA experiments were done with L-arginine (10^{-3}M) present. The amounts in perfusates collected per min in intervals 2-5 are expressed relative to those in interval 1. □, control; ■, L-NMMA; ▨, L-NMMA and L-arginine. *$p < 0.05$, significant change over time from values measured in interval 1. † $p < 0.05$, significant increase from the value in control perfusates in the same interval. In Figures 1-3, intervals 2, 3 and 5 are 10-min each and interval 4 is 30-min.

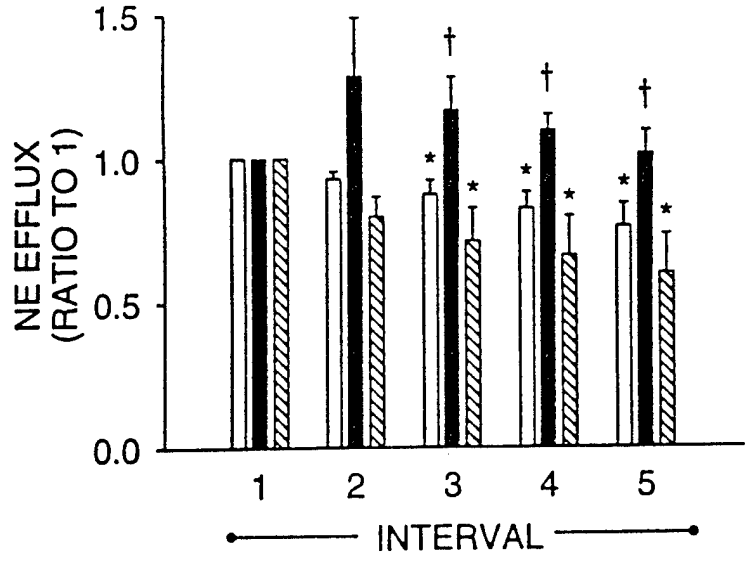


Figure 2. The effect of L-NMMA, 3 x 10^{-4}M, on the basal efflux of NE from dog adrenal glands. See legend to Fig. 1.

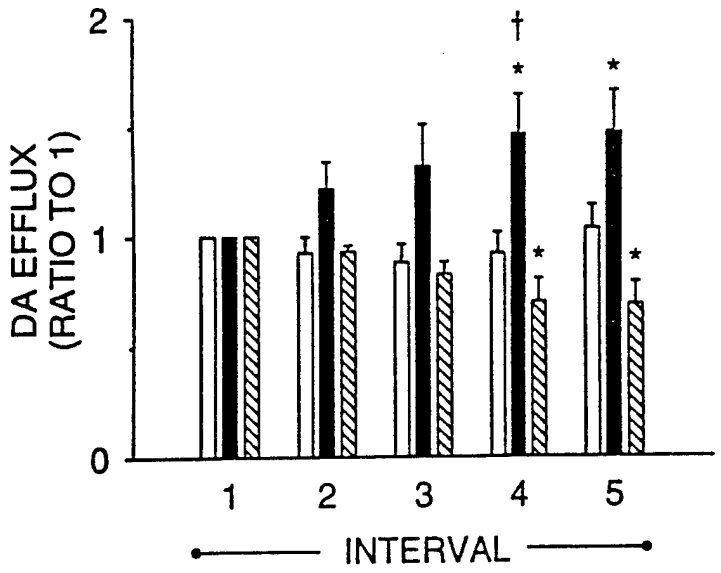


Figure 3. The effect of L-NMMA, 3 x 10^{-4}M, on the basal efflux of DA from dog adrenal glands. See legend to Fig. 1.

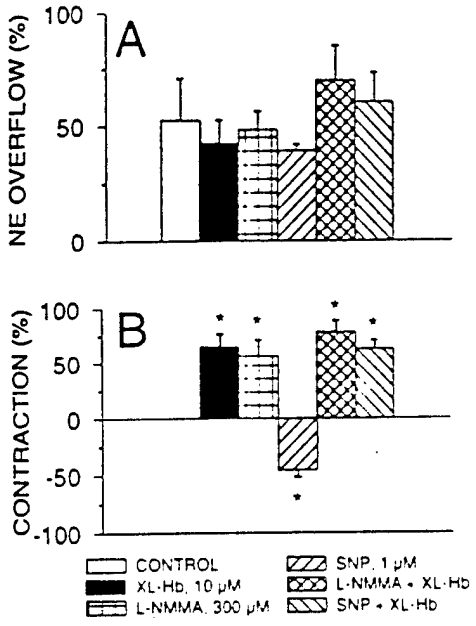


Figure 4. NE overflow (A) and contraction (B) during basal conditions in femoral artery strips superfused *in vitro*; effects of XL-Hb and of various treatments which modify tissue levels of NO. NE overflow was measured during a 5-min period and is expressed as the percentage of the NE which overflowed during a comparable 5-min period, 60 min prior to treatment. Contraction is expressed as the percentage of the contraction induced by stimulation of the vessel at 2 Hz prior to treatment. Values are ± SEM of determinations from five different experiments. *, Significant difference from corresponding value in control vessel, $P < 0.05$.

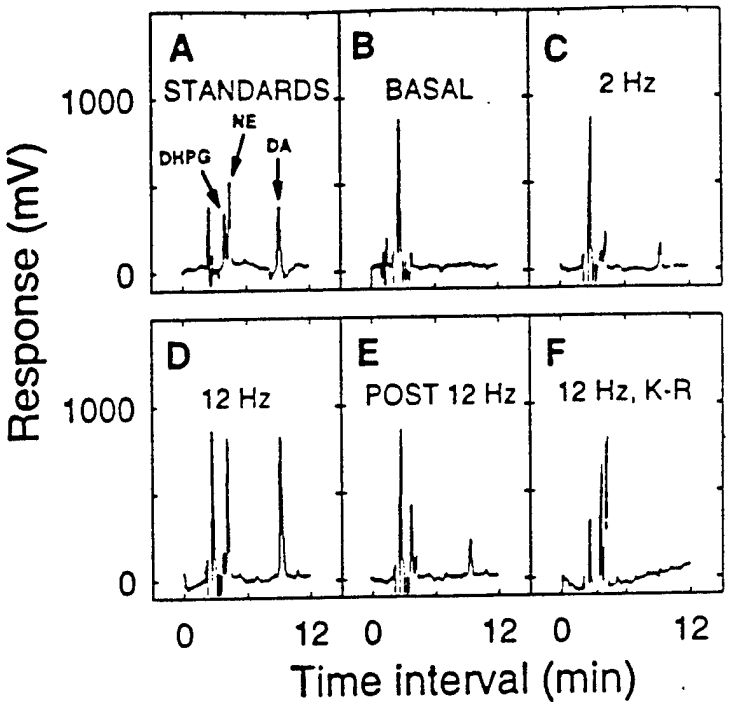


Figure 5. HPLC chromatograms illustrating the overflow of putative DA in mesenteric artery strips superfused *in vitro*.

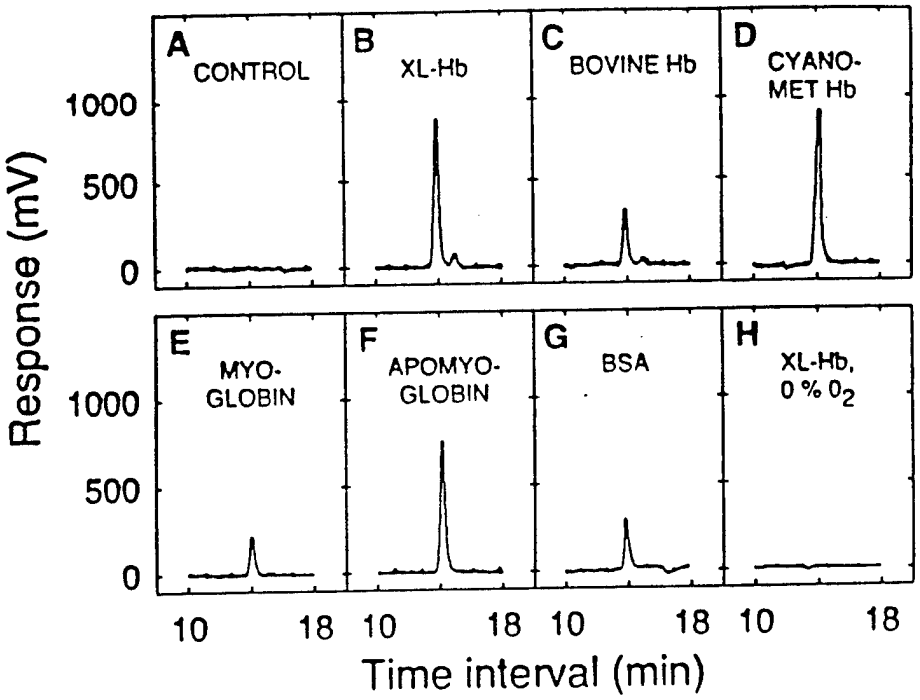


Figure 6. HPLC chromatograms scanned between 10 and 18 min, illustrating the production of an unknown compound which co-eluted with DA (see Figure 5) in the presence of various proteins.

PROJECT 3

"Effects of XL-Hgb Solution on Local and Baroreflex Control of Resistance"

Dr. M. J. Joyner

The studies conducted as a part of this project addressed issues related to in vivo vascular responses to administration of the vasodilating substances acetylcholine and nitroprusside inter-arterially. Twenty-four acutely instrumented anesthetized dogs were studied. Flow probes were placed around the left circumflex coronary artery, the mesenteric artery, and the femoral artery. These vessels have been selectively cannulated and the effects of increasing doses of acetylcholine (ACh, 1-100 mics/min) and nitroprusside (NTP, 1-100 mics/min) were studied. The animals also received systemic doses of alpha and beta blocking drugs to blunt any reflexogenic changes in arterial blood pressure in response to the various protocols. The first group of animals (n = 6) served as a time control, and successive dose response curves to ACh and NTP were conducted in the vessels of interest. These studies showed that the vasodilator responses to these compounds were stable over time. The second group of animals (n = 6) underwent an initial series of ACh and NTP dose response curves followed by rapid removal of roughly one-third of their blood volume. This volume was then replaced with albumin and the dose response curves repeated. The third group of animals underwent a similar bleeding protocol, but were resuscitated with an equal volume of cross-linked hemoglobin (~400-500 mls). The fourth group of animals (n = 6) underwent dose response curves during control conditions after treatment with the nitric oxide synthase inhibitor L-NMMA, and again after volume loading with cross-linked hemoglobin.

Administration of successive doses of acetylcholine resulted in an increase in blood flow from 2-500%, depending on the blood vessel (coronary ≥ femoral which was > mesenteric). Similar dose response relationships were observed with the nitroprusside administration. When no transfusion protocol was performed, these responses were stable with time and repeated dose response curves to both agents were nearly identical. In the second protocol, the blood flow (vasodilation) responses to ACh and nitroprusside were similar to those in group 1. With bleeding, there was a profound drop in arterial pressure from approximately 75-80 mmHg to 40-50 mmHg. This fall in pressure was promptly restored by administration of albumin. After albumin administration, dose response curves were again similar, and the reduced viscosity associated with a lower hematocrit appeared to have minimal effect on these relationships. In group 3, the pre-bleeding/transfusion responses were similar to the first two groups. The fall in arterial pressure with blood removal was similar to that observed in group 2. However, when volume resuscitation was performed with XL-Hgb, there was a prompt restoration in arterial blood pressure, and arterial pressure eventually rose to ~120 mmHg ($p < 0.05$ vs control). This rise in pressure was associated with vasoconstriction in the mesenteric and femoral beds. There was mild vasodilation in the coronary bed, perhaps as a result of the increased blood pressure and subsequent increased metabolic rate of the heart. Dose response curves to acetylcholine and nitroprusside were minimally affected in the mesenteric and femoral arteries. The changes in flow, conductance, or calculated resistance were blunted with

ACh administration in the coronary artery. The responses in the coronary artery to nitroprusside were unaffected by XL-Hgb. Table 1, along with figures 1 and 2, shows the effects of transfusion with XL-Hgb on the vasodilator responses to ACh and nitroprusside. Figure 3 shows the effects of L-NMMA followed by volume loading with XL-Hgb on the coronary dilator responses to ACh. Note the additive effect of the two treatments.

In group 4, the control dose response curves to both agents were again similar. Systemic administration of L-NMMA caused a 20-30 mm rise in arterial pressure. This blunted the ACh dose response curve in the coronary artery, but not the femoral and mesenteric arteries. With subsequent XL-Hgb administration, there was an additional increase in arterial pressure.

The following conclusions can be drawn from our work thus far.

1. Our animal preparation was stable with time, so the effects of any intervention were not due to time.

2. After acute bleeding and volume replacement with albumin, little change in the dose response relationships to ACh or nitroprusside were noted in the coronary, femoral, or mesenteric vascular beds.

3. Cross-linked hemoglobin caused marked systemic and pulmonary hypertension. This was accompanied by generalized vasoconstriction in all vascular beds studied. However, XL-Hgb only caused dramatic effects in the vasodilator responses to ACh and NTP in the coronary arteries.

4. Finally, XL-Hgb can cause hypertensive effects greater than those with L-NMMA on the basis of NO synthase blockade. This suggests, along with the failure of XL-Hgb to blunt NO-mediated vasodilation in response to various drug treatments, that other mechanisms may contribute to the vasoconstriction after XL-Hgb administration. Since these animals were under systemic alpha and beta blockades, it is unlikely that catecholamines could explain these effects. It would appear reasonable to suggest that either XL-Hgb has some sort of other vasoconstricting effect, or that renal mechanisms which can promote hypertension were activated after XL-Hgb administration.

5. Another possible explanation for the continued vasodilation observed when ACh is administered after XL-Hgb is that ACh may evoke release of a second vasodilating factor.

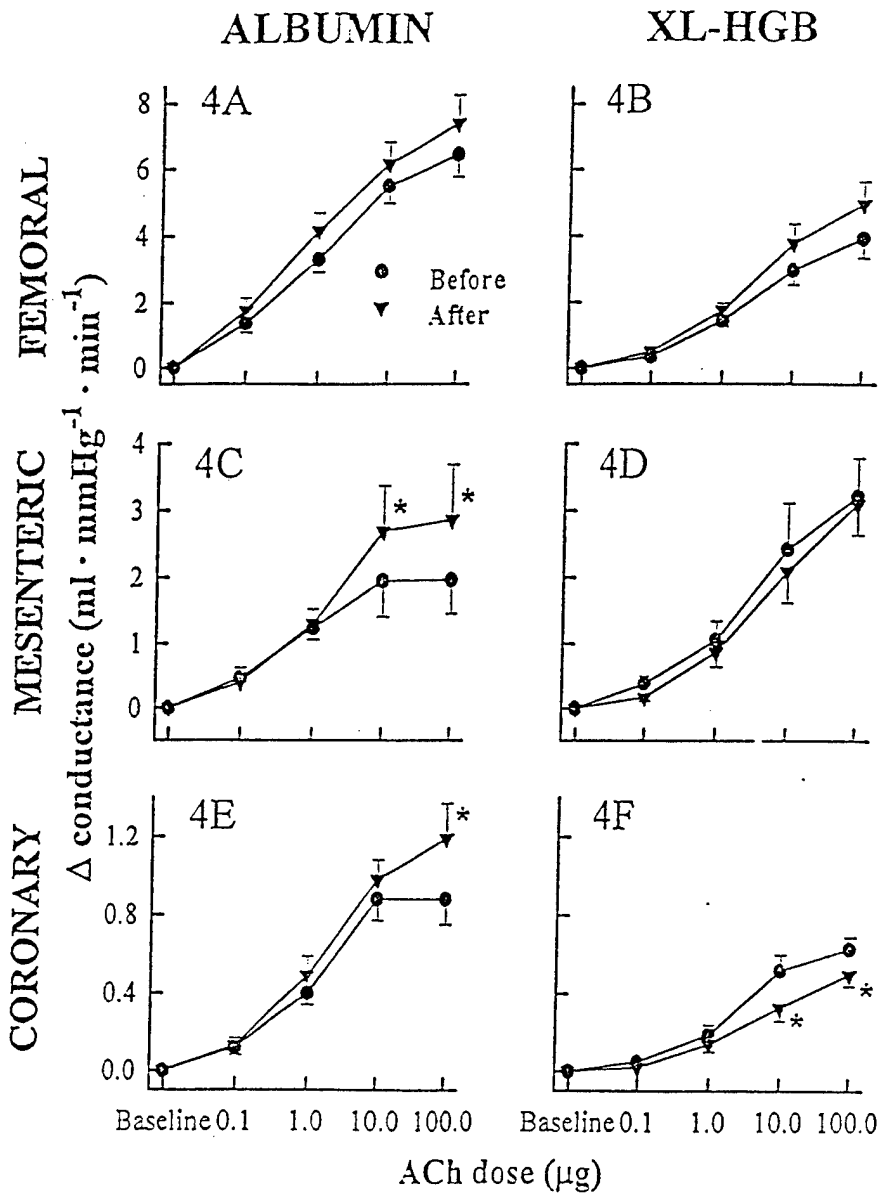


Figure 1. Dose-Response Curves to Acetylcholine Before and After XL-Hgb or Albumin

The change in conductance from baseline in response to various doses of acetylcholine (ACh) is shown for each arterial bed (femoral, mesenteric, and circumflex coronary) before and after partial (1/3 blood volume) exchange transfusion with either 5% albumin (n = 6) or XL-Hgb (n = 6) in an anesthetized canine preparation. There was no difference in the femoral artery responses to ACh in either the albumin (4A) or XL-Hgb (4B) conditions. In the mesenteric (4C) and coronary (4E) vascular beds after infusion of albumin, there was an increase in the response to higher doses of ACh. With XL-Hgb, there was no change in the dose-response relationships in the mesenteric vascular bed (4D); however, there was a decrease in the vasodilation in the coronary circulation (4F). *P < 0.05 versus pre-transfusion value.

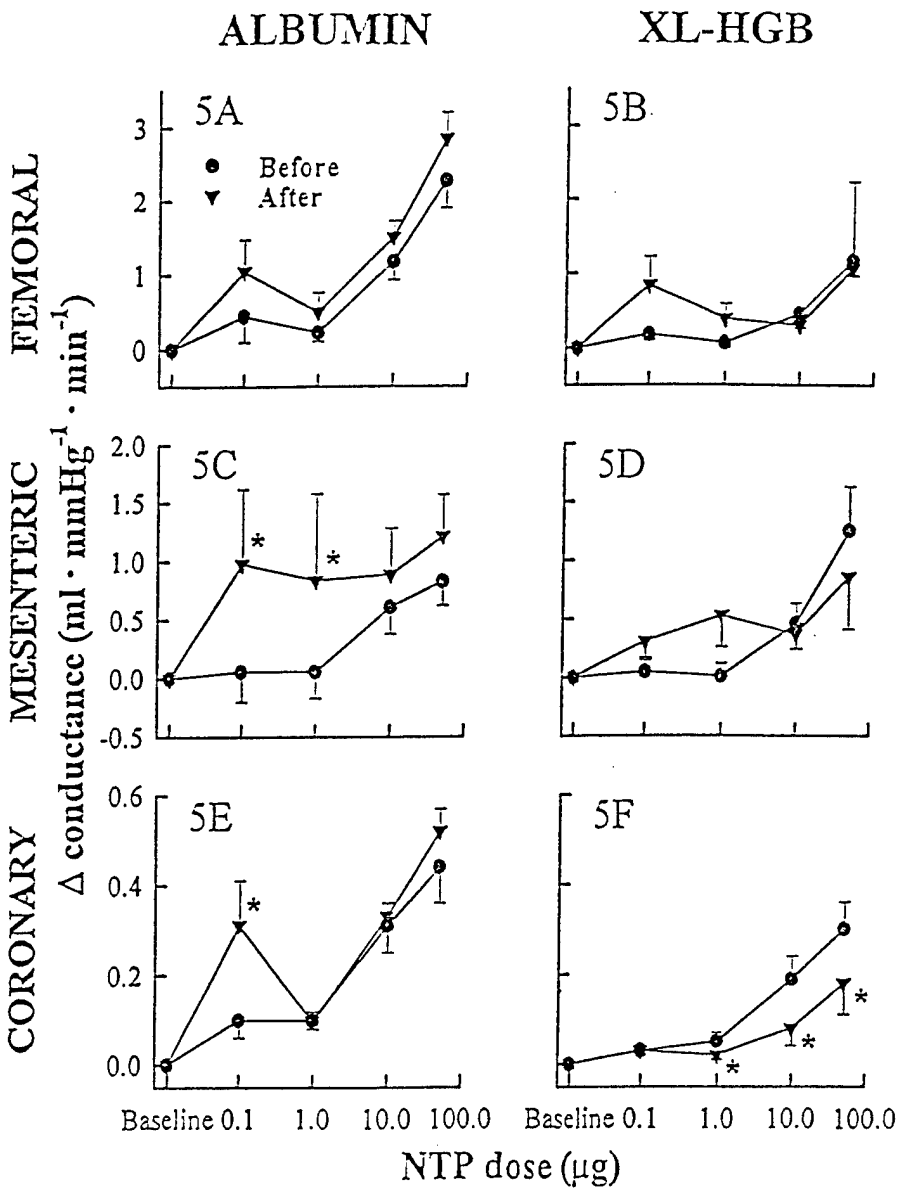


Figure 2. Dose-Response Curves to Sodium Nitroprusside Before and After XL-Hgb or Albumin

The change in conductance from baseline in response to various doses of sodium nitroprusside (NTP) is shown for each arterial bed (femoral, mesenteric, and circumflex coronary) before and after partial (1/3 blood volume) exchange transfusion with either 5% albumin (n = 6) or XL-Hgb (n = 6) in an anesthetized canine preparation. There were no changes in dose-response relationships in the femoral artery after either albumin (5A) or XL-Hgb (5B). There was increased conductance in the mesenteric vascular bed (5C) after albumin, but no change after XL-Hgb (5D). There was an increase in conductance in the coronary artery after albumin (5E) at the lower doses, and a decrease in conductance after XL-Hgb (5F). *P < 0.05 versus pre-transfusion value.

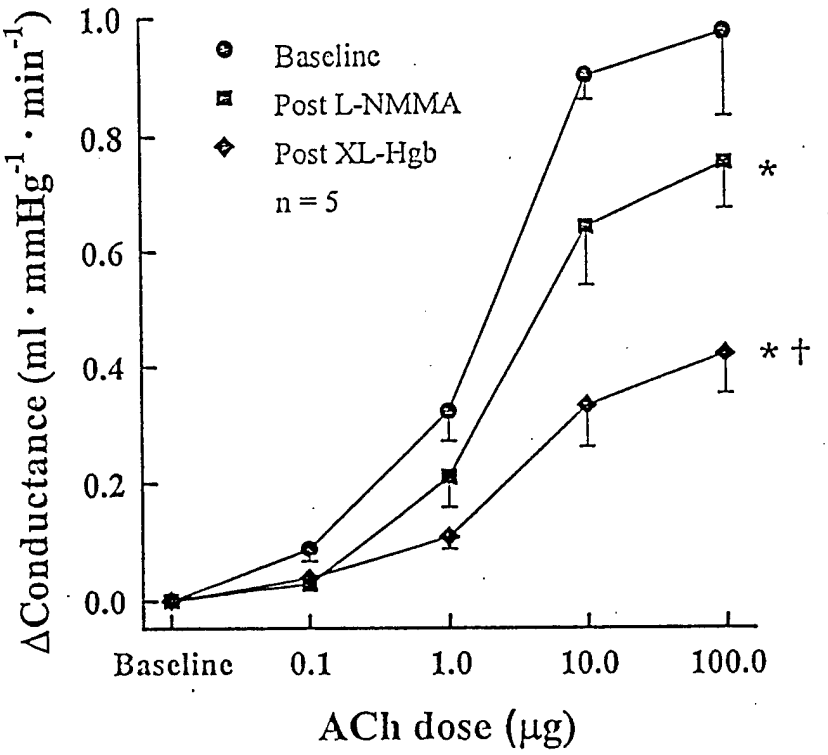


Figure 3. Dose-Response Curves to Acetylcholine After L-NMMA and the XL-Hgb

The change in conductance from baseline in response to various doses of acetylcholine (ACh) is shown for the circumflex coronary artery prior to drug administration, after administration of L-NMMA, and subsequently after 10% volume loading with XL-Hgb in an anesthetized canine preparation (n = 5). L-NMMA blunted the vasodilator responses to ACh and administration of XL-Hgb further blunted these vasodilator responses. *P < 0.05 versus pre-treatment values. +P < 0.05 versus post L-NMMA values.

Table. Baseline physiologic values for dogs in Protocol 1. Values are listed before and after partial exchange transfusion with either albumin (n = 6) or XL-Hgb (n = 6).

Group/Condition	Wt (kg)	Hgb ($gm \cdot dL^{-1}$) Blood/Plasma	MAP (mmHg)	CO ($L \cdot min^{-1}$)	pH	HR ($beats \cdot min^{-1}$)	Temp (°C)
Albumin							
Baseline	17.5±1.2	13.4±0.8 / 0	84±4	2.8±0.3	7.36±0.02	96±4	37.6±0.3
After transfusion	--	7.5±0.7* / 0	76±4*	4.3±0.6*	7.36±0.01	98±6	38.4±0.2
XL-Hgb							
Baseline	17.7±0.4	13.3±0.8* / 0	81±5	2.5±0.2	7.36±0.02	95±6	37.5±0.4
After transfusion	--	9.1±0.7* / 2.6±0.2*	112±8*	2.7±0.4	7.37±0.01	99±5	38.6±0.2

* = $P < 0.05$ versus baseline

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Dietz NM, Martin CM, Beltran-del-Rio AG, Joyner MJ. Effects of cross-linked hemoglobin on regional vascular conductance in dogs (in preparation).

PROJECT 4

"Effects of XL-Hgb Solution on Renal Blood Pressure Regulating Mechanisms"

Dr. J.C. Romero

X-Linked hemoglobin, a new type of blood substitute, may interact in mediating the natriuretic response to changes in pressure and volume. Elucidation of the mechanism of volume-induced natriuresis is important because it will provide insight into the pathogenesis of essential hypertension. For example, inhibition of nitric oxide synthesis increases mean arterial pressure which is accompanied by peripheral vasoconstriction and sodium retention. Likewise, a significant uptake of nitric oxide in the systemic circulation may lead to peripheral vasoconstriction of the vascular smooth muscle tissue. There is experimental evidence that the paramagnetic properties (odd number of electrons) of nitric oxide accounts for a high binding affinity for the heme iron complex. It is well known that hemoglobin binds nitric oxide producing a pronounced vasoconstriction. Recently, a different form of cross-linked hemoglobin (alpha-alpha with bis 3,5-dibromoscilicyl fumarate) has been synthesized with a chemical modification that increases the half-life of hemoglobin in the circulation, thus prolonging intravascular retention. The potential use of this compound as a blood substitute makes it important to understand its hemodynamic effects.

The first line of investigation, therefore, was undertaken to define the hemodynamic changes induced by the intravenous infusion of cross-linked hemoglobin (XL-Hb) on three vascular beds (iliac, mesenteric, and renal). Concomitant changes in blood pressure, glomerular filtration rate, and urinary sodium excretion were monitored. A similar volume-matched expansion with 6% Dextran was used as control, since its molecular weight is comparable to XL-Hb and it is biologically neutral. As shown in Figures 1 and 2, XL-Hb administration resulted in a significant decrease of blood flow to the three vascular beds coinciding with a significant increase in MAP. XL-Hb did not alter glomerular filtration rate or sodium excretion. In contrast, the administration of Dextran did not alter MAP but induced a significant increase of blood flow in the iliac, mesenteric, and renal vascular beds. These changes were accompanied by three-fold increases in sodium excretion.

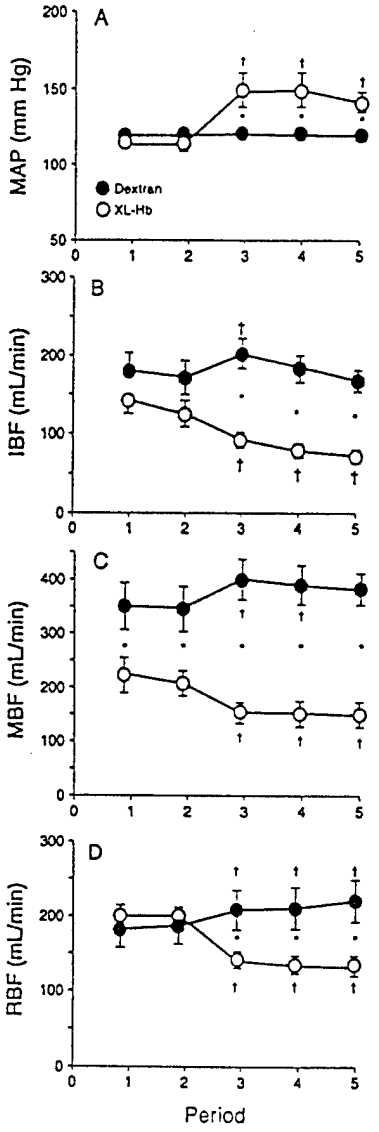

Figure 1a-d: Changes in mean arterial pressure (MAP) and in iliac (IBF), mesenteric (MBF), and renal (RBF) blood flows observed after the intravenous infusion of Dextran (closed circles) or cross-linked hemoglobin (XL-Hb) (open circles) during periods 2,3,4, and 5. Period 1 served as a baseline.

*$p<.05$ between the treatment groups

+$p<.05$ with respect to the basal period.

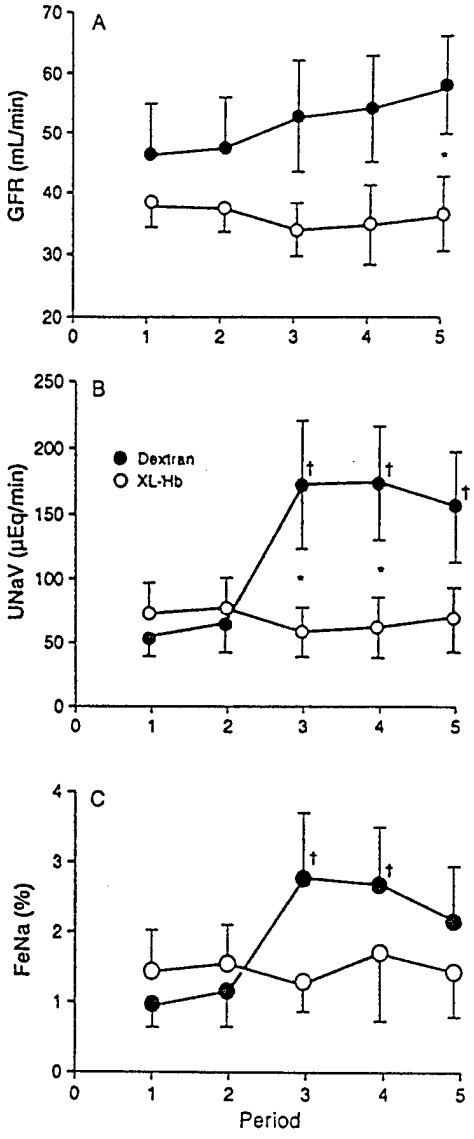

Figure 2a-c. Changes in glomerular filtration rate (GFR), urinary sodium excretion (UNaV) and fractional sodium excretion (FeNA) during the same conditions explained in the previous figure.

* $p<.05$ between the treatment groups

+ $p<.05$ with respect to the basal period.

These results demonstrate that XL-Hb administration is followed by hypertension, vasoconstriction, and blunted natriuresis. Such alterations cannot be attributed to volume expansion as they were not observed when a similar expansion was induced with Dextran. Therefore, these differences in the responses may be more related to specific biological actions of XL-Hb, such as its scavenging effects on nitric oxide.

The second line of investigation was to test the hypothesis that nitric oxide plays a role in vascular tone and regional blood flow regulation and to see if these changes parallel the effects of cross-linked hemoglobin. Mean arterial pressure, renal, mesenteric, and iliac blood flows, and cardiac output were measured in control and following progressive nitric oxide synthesis inhibition with L-NAME. As shown in Figure 3, at the highest dose (50 mg), there was an increase in mean arterial pressure, and a significant decrease of blood flow in the iliac, mesenteric, and renal blood flows. Glomerular filtration rate and urinary sodium excretion were not significantly different from control at the 50 mg dose of L-NAME (Figure 4).

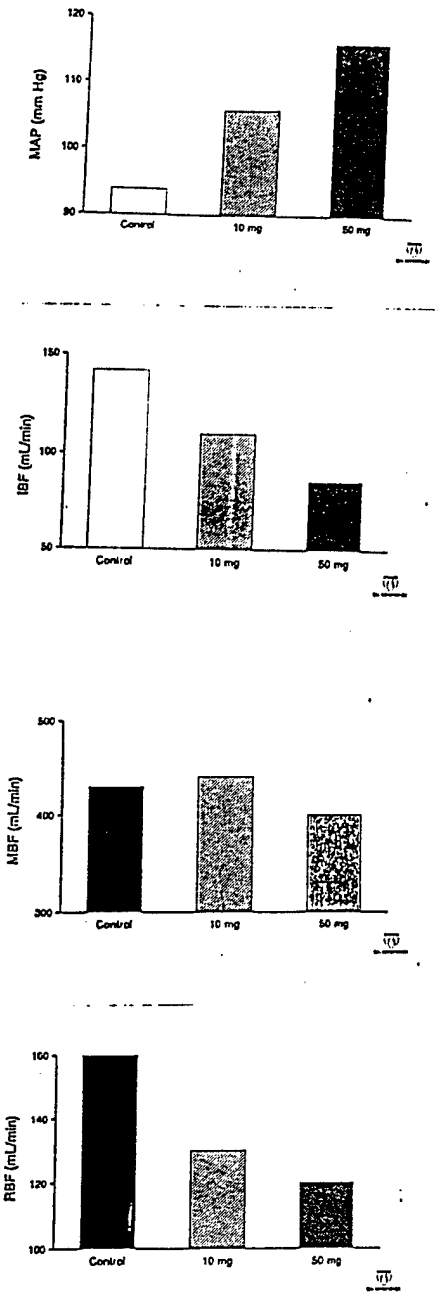

Figure 3 a-d. Response of mean arterial pressure (MAP) and in iliac (IBF), mesenteric (MBF), and renal (RBF) blood flows to the I.V. infusion of 10 and 50 mg/kg body weight (b.w.) of L-NAME in nine dogs.

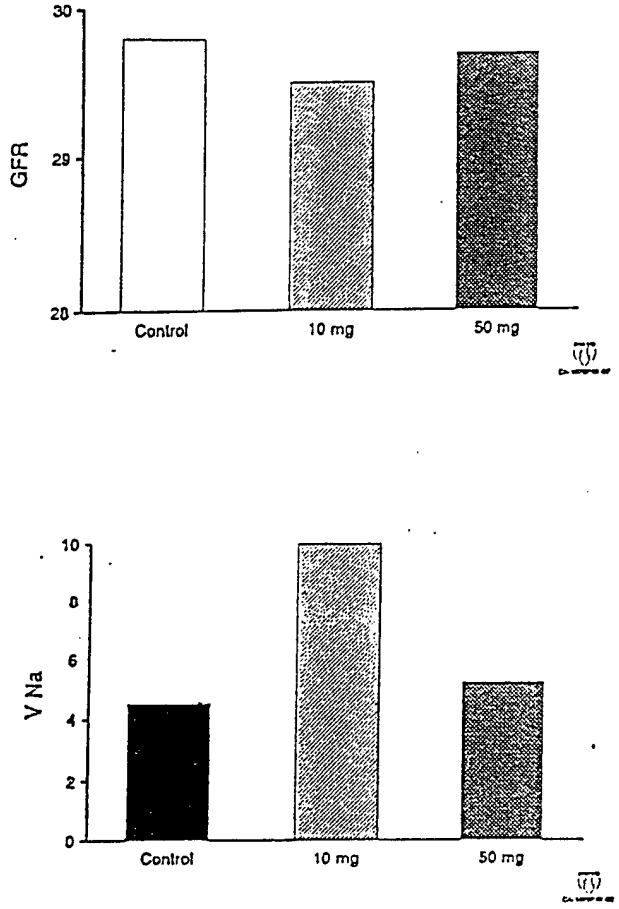

Figure 4 a-b. Response of glomerular filtration rate (GFR) and urinary sodium excretion (UNa) to the I.V. infusion of 10 and 50 mg/kg b.w. of L-NAME.

Changes in mean arterial pressure, blood flow to the three vascular beds, glomerular filtration rate, and urinary sodium excretion in response to the highest dose of L-NAME, paralleled the changes seen with the intravenous infusion of cross-linked hemoglobin. These findings support the notion that the biological actions of cross-linked hemoglobin may be related to its scavenging of nitric oxide.

The third line of investigation was to determine the compensatory effect of Dextran or cross-linked hemoglobin during hypovolemic hemorrhage on systemic vascular resistance, regional blood flow distribution and urinary sodium excretion. The dogs were hemorrhaged via the femoral artery a quantity of blood sufficient to reduce blood pressure by approximately 25%. Following this, an equal quantity of 6% Dextran of XL-Hb was infused intravenously and measurements were repeated. As seen in Table 1, in response to intravascular infusion of Dextran following hemorrhage, mean arterial pressure and blood flow to the iliac, mesenteric, and renal vascular beds were significantly increased.

Table 1. Renal responses to Dextran following hypovolemic hemorrhage

	CONTROL	HEMORRHAGE	DEXTRAN
MAP (mmHg)	137 ± 4	100 ± 5*	135 ± 4†
IBF (% of control)		-71 ± 4*	195 ± 26*
MBF (% of control)		-68 ± 1*	135 ± 41†
RBF (% of control)		-38 ± 6*	116 ± 25†
GFR (ml/min)	24 ± 4	10 ± 3*	41 ± 7†
FENa (%)	0.83 ± 0.2	0.24 ± .12*	1.25 ± .49

Values are means ± SE, n = 6 experiments. Abbreviations as in Figures 1 and 2.

*$p < .05$ compared with control period. †$p < .05$ compared with hemorrhage period.

As seen in Table 2, in response to XL-Hb, mean arterial pressure, blood flow to the iliac, mesenteric, and renal vascular beds, and urinary sodium excretion were all significantly increased. However, the response of blood flow to the three vascular beds was blunted compared with the response to Dextran infusion.

Table 2. Renal response to XL-Hb following hypovolemic hemorrhage

	CONTROL	HEMORRHAGE	XL-Hb
MAP (mmHg)	130 ± 3	100 ± 4*	130 ± 3†
IBF (% of control)		-70 ± 6*	66 ± 35†‡
MBF (% of control)		-53 ± 9*	84 ± 30†
RBF (% of control)		-35 ± 4*	27 ± 29†‡
GFR (ml/min)	22 ± 3	15 ± 4	25 ± 2†‡
FENa (%)	0.43 ± 0.20	0.10 ± .02*	3.13 ± 0.90*†‡

Values are means ± SE, n = 6 experiments. Abbreviations as in Table 1. *$p < .05$ compared with control period. †$p < .05$ compared with hemorrhage period. ‡$p < .05$ compared with Dextran period (group comparison).

These results are consistent with the previous observations that XL-Hb exerts a scavenging action on circulating NO.

More detailed information is provided on each of the studies in the enclosed manuscripts.

1. Cases A, Stulak JM, Romero JC. Hemodynamic and renal effects of cross-linked hemoglobin infusion. Am. J. Physiol. In review, 1996.

2. Cases A, Romero JC. Heterogenic vascular response to nitric oxide synthesis inhibition (abstract). Am. Heart Assoc., 1996.

3. Stulak JM, Cases A, Romero JC. Renal responses to Dextran during hypovolemic hemorrhage. In preparation, 1996.

CONCLUSIONS

The major conclusion of studies conducted under this contract are two-fold. First, cross-linked hemoglobin caused marked vasoconstriction and this constriction is due in part to the scavenging of nitric oxide by hemoglobin outside the red blood cell. Additionally, it appears reasonable to hypothesize that not all of the vasoconstriction and hypotension caused by administration of cross-linked hemoglobin can be accounted for solely on the basis of hemoglobin-scavenging nitric oxide. Some of the hypertensive effects may be due to entirely other mechanisms or may be secondary to other NO-mediated effects. For example, it appears that hemoglobin can cause vasoconstriction in a variety of vascular beds that is greater than that caused by nitric oxide synthase alone. This would suggest hemoglobin interferes with vasodilating pathways that are independent of nitric oxide. These conclusions are based on data from projects 1 and 3. Data from project 1 shows the XL hemoglobin interferes with vasodilating K^+ channel mechanisms. Data from project 3 shows that NO synthase blockade with L-NMMA can blunt endothelial-dependent dilation more than XL-Hgb alone. Additionally, hemoglobin transfusion also appears to blunt the normal natriuretic response seen during volume expansion in hypertension. This response mimics that seen during low level nitric oxide synthase inhibition in the kidney. This observation is consistent with the concept that intrarenal nitric oxide pathways play a role in the natriuretic and diuretic responses to volume expansion and hypertension. Finally, hemoglobin can also have modest effects on basal catecholamine release, which may be in part explained by its disruption of NO-mediated mechanisms. However, altered catecholamine release does not appear to account for the hypertension with XL-Hgb administration. In summary, cross-linked hemoglobin appears to scavenge NO and this scavenging of NO may account for 50% or more of its total vasoconstricting effect. In addition, NO-mediated hemoglobin-based vasoconstrictor mechanisms and/or vasoconstrictor mechanisms secondary to NO-scavenging appear to account for the remainder of the vasoconstriction.

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APPENDIX

PROJECT 1

"Effects of XL-Hgb on Endothelial Regulation of Vascular Tone"

Dr. Z.S. Katusic

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Crosslinked Hemoglobin Inhibits Endothelium-dependent Relaxations in Isolated Canine Arteries

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ABSTRACT. **1. Several previous *in vivo* studies demonstrated that crosslinked hemoglobin is a potent vasoconstrictor capable of significantly increasing arterial blood pressure following systemic administration. The precise mechanisms underlying the vascular effects of crosslinked hemoglobin are not clear. The present study was designed to determine the effect of crosslinked hemoglobin on the endothelial L-arginine-nitric oxide biosynthesis pathway in isolated canine arteries.**

2. Isolated femoral and renal arteries were suspended in organ chambers for isometric tension recordings. Endothelium-dependent relaxations to acetylcholine and calcium ionophore A23187 were studied in the absence or in the presence of crosslinked hemoglobin or hemoglobin. A radioimmunoassay technique was used to determine levels of guanosine 3′,5′-cyclic monophosphate (cyclic GMP) and adenosine 3′,5′-cyclic monophosphate (cyclic AMP).

3. A nitric oxide synthase inhibitor L-NAME (10^{-4}M) selectively inhibited endothelium-dependent relaxations to acetylcholine and calcium ionophore A23187. The inhibitory effect of L-NAME was reversed by L-arginine (3×10^{-4}M). Crosslinked hemoglobin (10^{-7}, 10^{-6} and 10^{-5}M) inhibited endothelium-dependent relaxations to acetylcholine (10^{-9}–10^{-5}M) or A23187 (10^{-9}–10^{-6}M). In the same concentration range, purified bovine hemoglobin exerted a similar inhibitory effect on relaxations mediated by activation of endothelial cells. Crosslinked hemoglobin (10^{-6}M) significantly reduced basal production of cyclic GMP, but did not affect production of cyclic AMP. Acetylcholine (10^{-6}M) stimulated production of cyclic GMP. This effect of acetylcholine was abolished in the presence of crosslinked hemoglobin.

4. These studies demonstrate that crosslinked hemoglobin impairs endothelium-dependent relaxations in isolated large conduit arteries. This effect appears to be mediated by the chemical antagonism of crosslinked hemoglobin against nitric oxide released from the endothelium. Inhibition of the endothelial L-arginine-nitric oxide biosynthesis pathway, with subsequent decrease of cyclic GMP in smooth muscle, may help to explain vasoconstrictor and pressor effects of crosslinked hemoglobin. GEN PHARMAC 27;2: 239–244, 1996.

KEY WORDS. Cyclic GMP, endothelium, nitric oxide, l-arginine

INTRODUCTION

The need for a safe and effective blood substitute has stimulated development of oxygen-carrying solutions that could be used in clinical conditions requiring blood transfusion (Winslow, 1992). Previous experiments demonstrated that a solution of human hemoglobin intermolecularly crosslinked with bis (3,5-dibromosalicyl) fumarate between α-chains (crosslinked hemoglobin), is capable of replacing blood and preserving cardiovascular functions (Hess *et al.*, 1991). Crosslinked hemoglobin is a potent vasoconstrictor and, in experimental animals as well as in humans, it may cause an increase in arterial blood pressure (Winslow, 1992; Schultz *et al.*, 1993; Motterlini and Macdonald, 1993; Sharma and Gulati, 1994).

The most likely mechanism responsible for the pressor effect of crosslinked hemoglobin is chemical inactivation of a potent endogenous vasodilator, nitric oxide. It is generally accepted that, under these conditions, unopposed vasoconstrictor stimuli increase arterial tone leading to hypertension. The high affinity of hemoglobin for nitric oxide has been utilized to characterize the chemical nature of endothelium-derived relaxing factor (Martin *et al.*, 1985). Furthermore, in our previous study, we used hemoglobin as a pharmacological tool to inhibit relaxations of cerebral arteries to exogenous or endogenous nitric oxide (Katušić *et al.*, 1989). A more recent report demonstrated that recombinant human hemoglobin inhibits endothelium-dependent relaxations to acetylcholine in the isolated rabbit aorta (Rioux *et al.*, 1994). This effect appears to be due to inactivation of nitric oxide released from the endothelium. However, effects of crosslinked hemoglobin on endothelial regulation of arterial tone have not been investigated in isolated blood vessels. The present study was designed to characterize the effects of crosslinked hemoglobin on endothelium-dependent relaxations and the L-arginine/nitric oxide pathway in large peripheral arteries.

MATERIALS AND METHODS

The experiments were performed on rings (3–5 mm in length) of femoral and renal arteries taken from dogs (15–20 kg) anesthetized with pentobarbital sodium (30 mg/kg IV) and euthanized *via* exanguination. The protocol was approved by the Institutional Animal Care and Use Committee. The arteries were placed in modified Krebs–Ringer bicarbonate solution [control solution (in mM): 118.3 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25.0 $NaHCO_3$, 0.026 calcium EDTA, and 11.1 glucose]. In certain rings, the endothelium was removed mechanically. Each ring was connected to an isometric force transducer (Gould MTC-2, Oxnard, CA, USA) and suspended in an organ chamber filled with 25 ml of control solution (37°C; pH = 7.4) and gassed with 94% O_2-6% CO_2. Isometric tension was recorded continuously.

The arteries were allowed to stabilize at a resting tension of 200–400 mg for 1 hr. Each ring was then gradually stretched to the optimal

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TABLE 1. Effect of L-arginine on endothelium-dependent relaxations to acetylcholine in femoral arteries treated with L-NAME

	Acetylcholine (-log M)						
	9	8.5	8	7.5	7	6.5	6
L-NAME (3×10^{-4}M)	22 ± 2	22 ± 2	22 ± 2	22 ± 2	24 ± 3	27 ± 3	33 ± 4
L-Arginine (10^{-3}M) + L-NAME (3×10^{-4}M)	24 ± 2	25 ± 1	26 ± 1	26 ± 1	34 ± 1	44 ± 6*	49 ± 7*

Values are means ± SEM (n = 7), and expressed as percent relaxation. *$P < 0.05$ as compared to L-NAME.

point of its length-tension curve as determined by the contractions to norepinephrine (3×10^{-7}M) (Katušić *et al.*, 1984). Optimal resting tensions were 12 *g* and 10 *g* for femoral and renal arteries, respectively (Katušić *et al.*, 1984). The functional integrity of endothelium was tested by the presence of relaxations to acetylcholine (10^{-6}M) (Katušić *et al.*, 1984).

Radioimmunoassay of cyclic nucleotides

A radioimmunoassay technique was used to determine the levels of cyclic 3',5'-guanosine monophosphate (cGMP) and cyclic 3',5'-adenosine monophosphate (cAMP) (Cosentino *et al.*, 1994). Rings with endothelium were initially incubated in control solution bubbled with 94% O_2-6% CO_2 gas mixture and kept at 37°C. After 1 hr, rings were incubated for an additional 30 min in a fresh solution containing indomethacin (10^{-5} M) and 3-isobutyl-1-methyl-xanthine (IBMX; 10^{-4}M) to inhibit the production of prostanoids and the degradation of cyclic nucleotides by phosphodiesterases, respectively. When crosslinked hemoglobin was used, it was present throughout the incubation period of 30 min. At the end of the experiments, rings were removed from the solution and frozen in liquid nitrogen. A radioimmunoassay kit (Amersham, Arlington Heights, IL, U.S.A.) was used to perform the measurements of cAMP and cGMP.

Drugs

The following pharmacological agents were used: acetylcholine hydrochloride (Sigma, St. Louis, MO, U.S.A.), L-arginine hydrochloride, calcium ionophore A23187, bovine, hemoglobin (Type 1), indomethacin, IBMX (all Sigma), molsidomine (SIN-1; Cassella, Frankfurt, Germany), N^G-nitro-L-arginine methylester (L-NAME), L-norepinephrine, papaverine hydrochloride (all Sigma) and pentobarbital sodium (Fort Dodge Laboratories, Fort Dodge, IA, U.S.A.). Stock solutions of the drugs were prepared fresh every day. Drugs were dissolved in distilled water such that volumes of <0.2 ml were added to the organ chambers. All concentrations are expressed as final molar (M) concentration in the bath solution.

Oxyhemoglobin was prepared by adding 10 mM sodium dithionate to a 1-mM solution of hemoglobin. The sodium dithionate was removed by dialysis in distilled water (containing 0.001% EDTA) for 2 hr at room temperature (Katušić *et al.*, 1989). The concentration of oxyhemoglobin in the final solution was determined by Co-oximeter Analyzer (IL482, Instrumentation Laboratries, Lexington, MA, U.S.A.). Crosslinked hemoglobin was obtained from the Walter Reed Army Institute of Research, Washington, DC, USA. The solution was prepared from stroma-free human hemoglobin from outdated blood modified with bis (3,5-dibromosalicyl) fumarate according to the method of Snyder *et al.* (1987). The crosslinked hemoglobin was formulated in Ringer acetate (7 g/100 ml) and maintained at 4°C until the day of use. At that time, it was passed through a 0.22 μm filter to remove particulate matter, then warmed to 37°C by placing the bag in a water bath.

The incubation time was 30 min for hemoglobin, crosslinked hemoglobin, and indomethacin, 5 min for L-arginine, and 15 min for L-NAME.

Concentration-response curves were obtained in a cumulative fashion. Several rings cut from the same artery were studied in parallel; only one concentration-response curve was made per preparation. The relaxations were expressed as a percentage of maximal relaxations to papaverine (3×10^{-4}M).

Statistical Analysis

The results are expressed as means ± SE; n refers to the number of dogs. Statistical evaluation of the data was performed by Student's t-test for paired observations. $P<0.05$ was considered to be statistically significant.

RESULTS

Effect of L-NAME on endothelium-dependent relaxations to acetylcholine and A23187

During contractions induced with norepinephrine (3×10^{-7}M), acetylcholine and calcium ionophore A23187 caused endothelium-dependent relaxations in canine femoral arteries. L-NAME (3×10^{-4}M) abolished relaxations to acetylcholine (Fig. 1), and significantly reduced relaxations to A23187 ($n=9$, data not shown). The inhibitory effect of L-NAME was partially reversed in the presence of L-arginine (10^{-3}M; Table 1). In femoral arteries without endothelium, L-NAME did not affect relaxations to the nitric oxide donor SIN-1 ($n=7$, data not shown).

Effect of crosslinked hemoglobin on endothelium-dependent relaxations to acetylcholine and A23187

In femoral arteries, increasing concentrations of crosslinked hemoglobin (10^{-7}, 10^{-6} and 10^{-5}M) caused concentration-dependent inhibition of endothelium-dependent relaxations to acetylcholine (Fig. 2). Crosslinked hemoglobin also significantly reduced relaxations to A23187 (Fig. 3). The inhibitory effect of crosslinked hemoglobin on relaxations to A23187 was not concentration-dependent.

In renal arteries, crosslinked hemoglobin (10^{-7}M) significantly reduced endothelium-dependent relaxations to acetylcholine as well as to A23187 (Figs. 4 and 5).

Effects of hemoglobin on endothelium-dependent relaxations to acetylcholine and A23187

In femoral arteries, as well as in renal arteries, hemoglobin significantly reduced endothelium-dependent relaxations to acetylcholine (Figs. 6 and 7).

Effect of crosslinked hemoglobin on production of cyclic nucleotides

In arteries with intact endothelial cells, acetylcholine (10^{-6}M) caused significant increase in cyclic GMP production (Table 2). In contrast,

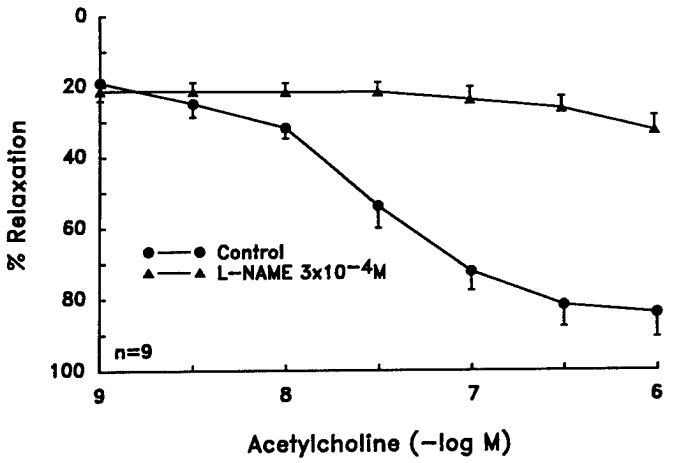

FIGURE 1. Concentration-response curves to acetylcholine in canine femoral arteries with endothelium in the absence and presence of L-NAME (3×10^{-4}M) are shown. Relaxations were obtained during contractions to norepinephrine (3×10^{-7}M). Data are shown as means±SE and are expressed as percent of maximal relaxation induced by papaverine (3×10^{-4}M; 100% = 15.6±3.1 g, 19.8±1.4 g, n = 9, for control rings and in the presence of L-NAME, respectively). Difference between control rings and rings treated with L-NAME is statistically significant (P<0.05).

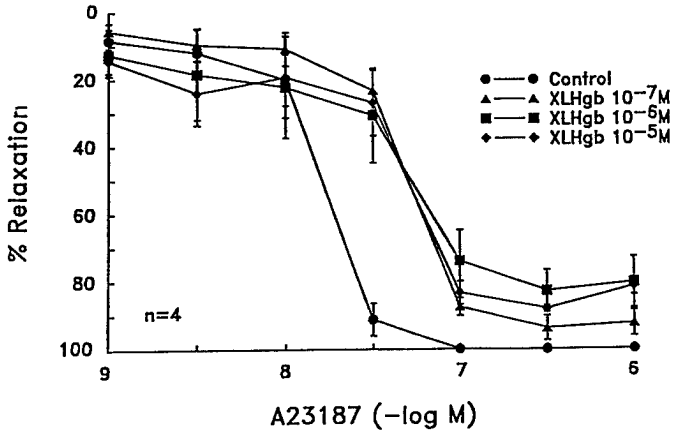

FIGURE 3. Concentration-response curves to A23187 in canine femoral arteries with endothelium in the absence and presence of crosslinked hemoglobin (XLHgb) are shown. Relaxations were obtained during contractions to norepinephrine (3×10^{-7}M). Data are shown as means±SE and are expressed as percent of maximal relaxation induced by papaverine (3×10^{-4}M; 100% = 6.8±1.9 g, 6.1±2.3 g, 5.1±1.6 g, and 6.2±2.0 g, n = 4, for control rings and in the presence of 10^{-7}, 10^{-6} and 10^{-5}M XLHgb, respectively). Difference between control rings and rings treated with crosslinked hemoglobin is statistically significant (P<0.05).

it did not affect production of cyclic AMP (n=4, data not shown). In the presence of crosslinked hemoglobin (10^{-6}M), basal production of cyclic GMP (but not cyclic AMP) was significantly reduced in femoral and renal arteries. Crosslinked hemoglobin also abolished stimulatory effect of acetylcholine on production of cyclic GMP (Table 2).

DISCUSSION

The present study demonstrates that, in isolated canine peripheral arteries, crosslinked hemoglobin inhibits endothelium-dependent relaxations. The effect of crosslinked hemoglobin is apparently mediated by inactivation of L-arginine/nitric oxide pathway. This conclusion is supported by several lines of evidence: (a) endothelium-dependent relaxations to acetylcholine and A23187 were selectively impaired in the presence of a nitric oxide synthase inhibitor L-NAME, (b) L-arginine was able to partially reverse the inhibitory effect of L-NAME, (c) crosslinked hemoglobin and hemoglobin inhibited endotheliumdependent relaxations to acetylcholine and A23187, and (d) the inhibitory effect

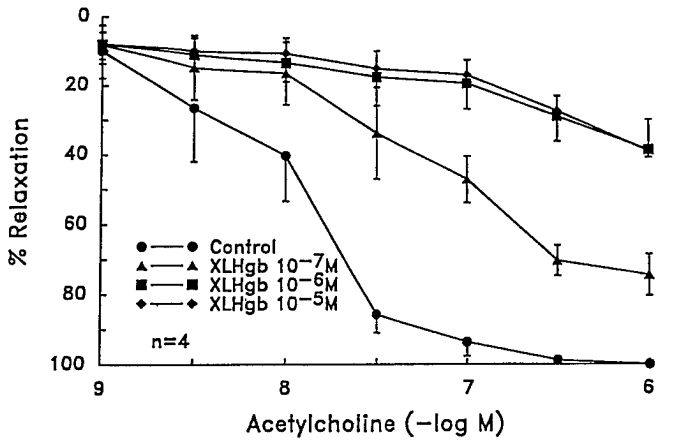

FIGURE 2. Concentration-response curves to acetylcholine in canine femoral arteries with endothelium in the absence and presence of crosslinked hemoglobin (XLHgb) are shown. Relaxations were obtained during contractions to norepinephrine (3×10^{-7} M). Data are shown as means±SE and are expressed as percent of maximal relaxation induced by papaverine (3×10^{-4} M; 100% = 11.8±1.5 g, 15.8±0.6 g, 13.6±2.6 g, and 16.8±1.9 g, n = 4, for control rings and in the presence of 10^{-7}, 10^{-6} and 10^{-5} M XLHgb, respectively). Difference between control rings and rings treated with crosslinked hemoglobin is statistically significant (P<0.05).

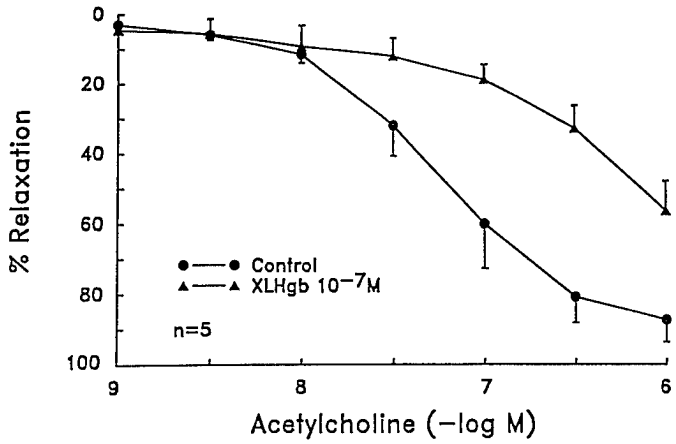

FIGURE 4. Concentration-response curves to acetylcholine in canine renal arteries with endothelium in the absence and presence of crosslinked hemoglobin (XLHgb). Relaxations were obtained during contractions to norepinephrine (3×10^{-7}M). Data are shown as means±SE and are expressed as percent of maximal relaxation induced by papaverine (3×10^{-4}M; 100% = 8.2±0.6 g, 10.0±1.6 g, n = 5, for control rings and in the presence of XLHgb, respectively). Difference between control rings and rings treated with crosslinked hemoglobin is statistically significant (P<0.05).

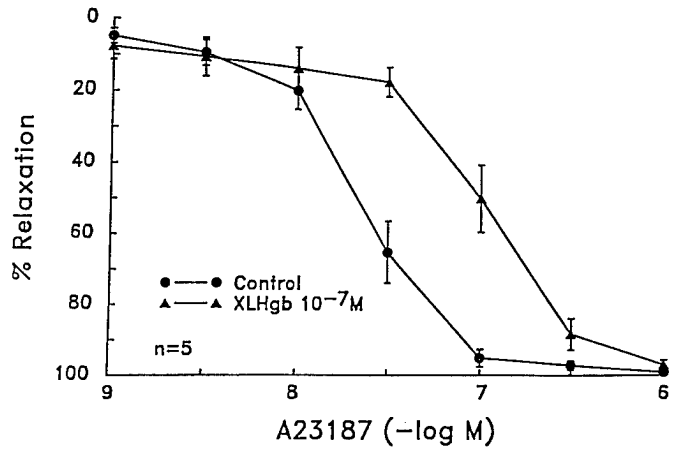

FIGURE 5. Concentration-response curves to A23187 in canine renal arteries with endothelium in the absence and presence of crosslinked hemoglobin (XLHgb). Relaxations were obtained during contractions to norepinephrine (3×10^{-7}M). Data are shown as means±SE and are expressed as percent of maximal relaxation induced by papaverine (3×10^{-4}M; 100%=5.9±1.0 g, 6.8±1.3 g, $n=5$, for control rings and in the presence of XLHgb, respectively). Difference between control rings and rings treated with crosslinked hemoglobin is statistically significant ($P<0.05$).

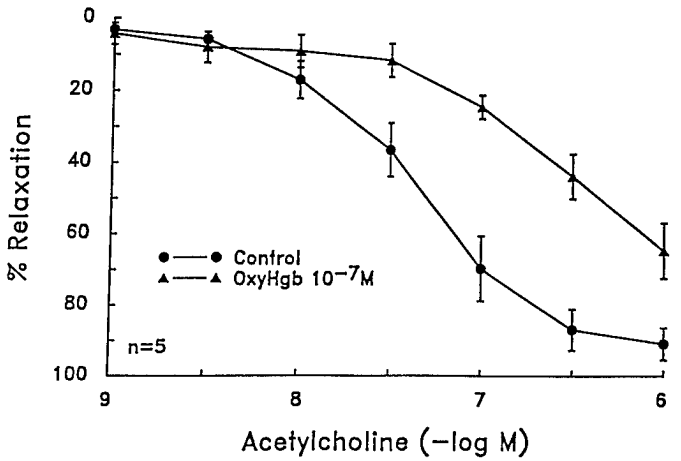

FIGURE 7. Concentration-response curves to acetylcholine in canine renal arteries with endothelium in the absence and presence of hemoglobin (OxyHgb). Relaxations were obtained during contractions to norepinephrine (3×10^{-7}M). Data are shown means±SE and expressed as percent maximal relaxation induced by papaverine (3×10^{-4}M; 100%=8.9±0.5 g, 8.8±0.5 g, $n=5$, for control rings and in thepresence of 10^{-7}M OxyHb, respectively). Difference between control rings and rings treated with OxyHgb is statistically significant $P<0.05$).

of crosslinked hemoglobin was associated with decreased production of cyclic GMP under basal conditions and during stimualtion of endothelial cells with acetylcholine.

The results of our study confirmed previous findings demonstrating the inhibitory effect of L-NAME on endothelium-dependent relaxations in isolated arteries (Cosentino *et al.*, 1993). The effect of L-NAME could be due to inhibition of nitric oxide synthase (Cosentino *et al.*, 1993), muscarinic receptor antagonism (Buxton *et al.*, 1993), or nonselective inhibition of smooth muscle relaxation. L-NAME did not affect relaxations to SIN-1, indicating that it has selectivity for relaxations induced by activation of the endothelial cells. The contribution of the antimuscarinic effect of L-NAME cannot be estimated from our experiments. However, the ability of L-arginine to partially restore relaxations inhibited with L-NAME, as well as the fact that acetylcholine caused significant increase in cyclic GMP levels, demonstrates that, in femoral and renal arteries, endothelium-dependent relaxations to muscarinic activation are mediated by production of nitric oxide. Our previous study on cerebral arteries demonstrated that L-NAME selectively inhibits endothelium-dependent relaxations and production of cyclic GMP (Cosentino *et al.*, 1994), further supporting our conclusion that the effect of L-NAME is due to inhibition of nitric oxide synthase.

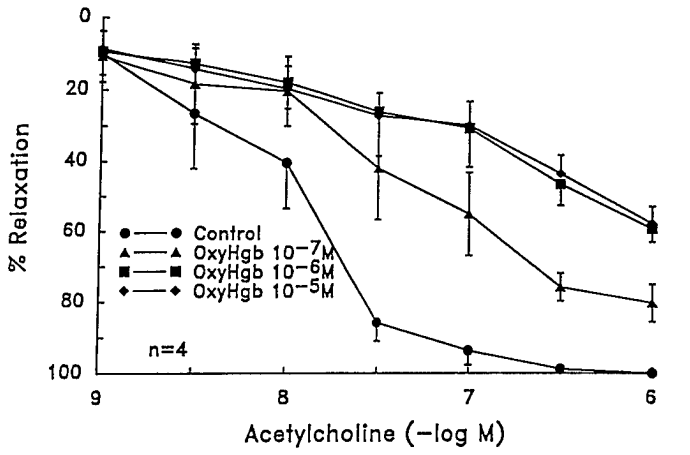

FIGURE 6. Concentration-response curves to acetylcholine in canine femoral arteries with endothelium in the absence and presence of hemoglobin (OxyHgb). Relaxations were obtained during contractions to norepinephrine (3×10^{-7}M). Data are shown means±SE and expressed as percent maximal relaxation induced by papaverine (3×10^{-4}M; 100%=11.8±1.5 g, 15.6±2.5 g, 14.8±1.0 g and 14.3±2.6 g, $n=4$, for control rings and in the presence of 10^{-7}, 10^{-6} and 10^{-5}M OxyHgb, respectively). Difference between control rings and rings treated with OxyHgb is statistically significant ($P<0.05$).

The ability of mammalian hemoglobin to bind nitric oxide has been described by Gibson and Roughton in 1957. This chararacteristic of the hemoglobin molecule has been extensively used in analysis of mechanisms of endothelium-dependent relaxations (Katušić *et al.*, 1989; Lüscher and Vanhoutte, 1990). The results of our study demonstrate that chemical modification of hemoglobin molecule by crosslinking with bis(3,5-dibromosalicyl) fumarate does not affect its ability to inhibit these relaxations. In addition, similar inhibition of endothelium-dependent relaxations was obtained with identical concentrations of crosslinked hemoglobin and hemoglobin, confirming that crosslinking does not decrease affinity of the hemoglobin molecule for nitric oxide (Alayash *et al.*, 1993).

In femoral and renal arteries, measurements of cyclic nucleotide levels in the presence of crosslinked hemoglobin revealed inhibition of cyclic GMP production. Crosslinked hemoglobin did not affect production of cyclic AMP, demonstrating that, in concentrations up to 10^{-6}M, it has a selective inhibitory effct on cyclic GMP production. It is well established that cyclic GMP is a second messenger responsible for relaxations of blood vessels induced by nitric oxide (Ignarro *et al.*, 1987; Moncada *et al.*, 1991). As expected, acetylcholine significantly increased cyclic GMP in the arterial wall. The stimulatory effect of acetylcholine on cyclic GMP was abolished by crosslinked hemoglobin. It is also important to note that crosslinked hemoglobin significantly decreased production of cyclic GMP under basal conditions. These findings dem-

TABLE 2. The effect of crosslinked hemoglobin (XLHb) on baseline and acetylcholine (ACh)-induced production of cyclic GMP in arteries with endothelium

	Control	ACh (10^{-6}M)	XLHb (10^{-6}M)	XLHb (10^{-6}M) + AC (10^{-6}M)
Femoral artery	19.0 ± 19.3	60.2 ± 43.8*	2.8 ± 3.2**	6.1 ± 6.4**
Renal artery	10.6 ± 6.3	39.0 ± 33.0*	3.0 ± 2.7**	2.2 ± 2.9**

Values are means ± SEM (n = 9), expressed as pmol/g wet weight. *$P < 0.05$, significantly different from control; **$P < 0.05$, significantly different from control and arteries treated with ACh.

onstrate that, in large canine arteries, crosslinked hemoglobin may impair function of the endothelial L-arginine pathway. Impaired vascular endothelial function may have important implications, not only for regulation of arterial tone, but for interaction between endothelium and circulating blood cells, including platelets and leukocytes (Lüscher and Vanhoutte, 1990; Katušić, 1994). Whether or not administration of crosslinked hemoglobin *in vivo* may favor aggregation of platelets or leukocyte adhesion remains to be determined.

A number of previous studies have demonstrated that endothelium-dependent relaxations to A23187 are due to translocation of calcium into endothelium, with subsequent activation of nitric oxide synthase (Lüscher and Vanhoutte, 1990). In isolated canine peripheral and cerebral arteries, this effect is associated with a significant increase in cyclic GMP levels in smooth muscle cells (Cosentino *et al.*, 1994; Katušić *et al.*, unpublihsed). In our experiments, relaxations to calcium ionophore were reduced in the presence of crosslinked hemoglobin in femoral and renal arteries. This finding is best explained by the ability of crosslinked hemoglobin to inactivate nitric oxide released from endothelial cells. It also supports our conclusion that the inhibitory effect of crosslinked hemoglobin on endothelium-dependent relaxations to acetylcholine is due to interactions of hemoglobin molecule with released nitric oxide, rather than to an effect on the cell membrane or muscarinic receptors. However, in femoral and renal arteries and unlike acetylcholine, the maximal effect of A23187 was not reduced with high concentrations of crosslinked hemoglobin. The reason for this difference is not clear. One possible explanation could be related to the fact that A23187 may activate endothelial cells to release relaxing factors other than nitric oxide (prostanoids or hyperpolarizing factor; Lüscher and Vanhoutte, 1990). The results of the present study do not allow any conclusion with regard to the mechanism of endothelium-dependent relaxations to A23187 resistant to the inhibitory effect of crosslinked hemoglobin.

Our results clearly show that crosslinked hemoglobin, developed for use as a blood substitute, inhibits endothelium-dependent relaxations in isolated large conduit canine arteries. These results are in agreement with the previously reported ability of purified hemoglobin and recombinant hemoglobin to act as chemical antagonists against vascular endothelial nitric oxide (Katušić *et al.*, 1989; Rioux *et al.*, 1994). Our study expands previous findings providing biochemical data to demonstrate that production of cyclic GMP is abolished in isolated arteries exposed to crosslinked hemoglobin. The impairment of endothelial L-arginine pathway function certainly may help to explain the increase in arterial blood pressure observed following systemic administration of crosslinked hemoglobin solution. However, because a pressor effect observed *in vivo* primarily reflects vasoconstriction of arterioles, further experiments on isolated small resistance blood vessels are required to characterize the mechanisms responsible for the increase in vascular tone induced by crosslinked hemoglobin.

What are the possible clinical implications of our findings? It is clear that crosslinked hemoglobin-based solutions have the potential to impair function of the L-arginine pathway in vascular endothelium. The major acute consequences of this biochemical defect include vasoconstriction, increased aggregation of platelets, and increased adhesion of leukocytes (Katušić, 1994; Shepherd and Katušić, 1991). These effects may have serious consequences in patients with coronary artery disease, hypertension, hypercholesterolemia, or diabetes. In all of these conditions, production and/or activity of nitric oxide released from the endothelium is already decreased, and a much smaller amount of circulating free hemoglobin than reported in our study may be required to abolish the function of endogenous nitric oxide. In that regard, it will be very important to define the vascular effects of hemoglobin-based blood substitutes, not only in healthy animals, but in experimental models of different cardiovascular diseases. These studies will certainly help to more accurately predict possible adverse effects of hemoglobin solutions in clinical conditions associated with the dysfunction of vascular endothelial cells.

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HEMODYNAMIC AND RENAL EFFECTS OF CROSS-LINKED HEMOGLOBIN INFUSION

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Running Title: Cross-linked hemoglobin administration in dogs

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ABSTRACT

It is well known that hemoglobin binds nitric oxide producing a pronounced vasoconstriction in isolated arteries. However, it is debatable whether or not such an effect takes place in whole animals, because hemoglobin also is known to catalyze the formation of prostaglandins from arachidonic acid. Acute studies were performed to evaluate the effects induced by intravenous infusion of cross-linked hemoglobin (XL-Hb) on blood pressure and renal, iliac, and mesenteric flows, as well as on renal function in 6 anesthetized dogs. A similar volume-matched expansion with 6% Dextran was used as control (n=6). Glomerular filtration rate (GFR), urinary flow, and total and fractional sodium excretion were measured before and after XL-Hb or dextran infusion to evaluate possible renal function changes. XL-Hb administration resulted in a 29 % elevation in BP and a significant decrease of blood flow (30-37%) to the three vascular beds. XL-Hb did not alter GFR or sodium excretion, despite the increase in BP. In contrast, the administration of Dextran did not significantly alter BP but induced a significant increase (6-13%) of blood flow in the three vascular beds. These changes were accompanied by three-fold increases in urinary flow and sodium excretion without alterations in GFR. The binding effect of XL-Hb on NO was studied in isolated renal arteries in organ chambers. These *in vitro* studies demonstrated that XL-Hb blunted the endothelium-mediated vasodilator response to the calcium ionophore A23187 and to acetylcholine. Our results demonstrate that XL-Hb administration is followed by hypertension, vasoconstriction and blunted natriuresis. All these effects are compatible with the scavenging effect on NO attributed to XL-Hb.

Index Terms: Dextran, nitric oxide, prostaglandins

INTRODUCTION

It is well known that the paramagnetic properties (odd number of electrons) of nitric oxide (NO) account for a remarkable binding affinity for the heme iron complex (8). Such characteristic accounts for both the NO-activation of guanylate cyclase as NO binds the heme group of this enzyme, and the inactivation of NO by hemoglobin (Hb) (1). This later effect has been well described in isolated arteries, but it has never been explored in whole animals (9,10). From a speculative point of view, a significant uptake of NO in systemic circulation may lead to a vasoconstriction if the binding to Hb imposed a reduction on the amount of endothelial NO which diffuses towards the vascular smooth muscle. However, there are also experimental evidences showing that Hb catalyzes the transformation of arachidonic acid to prostaglandins with remarkable specificity (3,4,16,22). Such a cyclooxygenase-like activity could stimulate the formation of vasodilators, such as PGI_2 or PGE_2 (25-26), which may decrease systemic blood pressure. This effect would counteract the vasopressor action of NO suppression.

Until recently, the possibility of testing the validity of these assumptions was precluded by the instability and rapid breakdown of stroma-free hemoglobin. Such a problem has been recently overcome by the synthesis of different forms of cross-linked hemoglobin (XL-Hb). One of these compounds is hemoglobin cross-link alpha-alpha with bis (3,5-dibromosalicyl) fumarate (7). This chemical modification increases the half-life of Hb in circulation and reduces its renal clearance, thus prolonging intravascular retention (7). As it is apparent, the potential clinical use of this compound as a blood substitute (8,24) creates an additional interest in studying the hemodynamic effects of free hemoglobin in circulation.

It should be mentioned here that Shultz et al (20) showed that the intravenous of administration of diaspirin cross-linked hemoglobin to Sprague Dowley rats produced a transient increase of blood pressure. However, no attempts were made in this study to determine if a vasoconstrictor effect of cross-linked hemoglobin were uniformly exerted in different vascular beds or which were the specific changes produce by cross-linked hemoglobin in renal function and urine sodium excretion. Such information on extracellular fluid volume homeostasis is very critical when

evaluating the characteristics of a volume expander such as cross-linked hemoglobin.

This study was therefore undertaken to define the hemodynamic changes induced by the intravenous infusion of XL-Hb on three vascular beds: iliac, mesenteric, and renal. These vascular beds were selected as they are important contributors of total peripheral resistance (5) and their diversity in metabolic activities justify exploring different responses depending on NO and/or PG's involvement. In these studies, the concomitant changes in blood pressure and renal excretory function, namely glomerular filtration rate and urinary sodium excretion, were also monitored. The results of these studies were compared to the hemodynamic effects produced by equiosmolar concentrations of dextran. This substance was chosen over whole blood, plasma or albumin because its molecular weight is comparable to that of XL-Hb and it is biologically neutral. This characteristic help to distinguish the hemodynamic effects that could be derived from the XL-Hb-induced volume expansion, exempted from its biological effects.

To determine if XL-Hb produces the same vasoconstriction than that attributed to the NO scavenging actions of hemoglobin, we characterized the effects of XL-Hb on the relaxation induced by either calcium ionophore A23187 or acetylcholine in isolated renal arteries, which are maneuvers that stimulate the synthesis of NO.

MATERIALS AND METHODS

Intravenous infusion of XL-Hb or dextran

Twelve male mongrel dogs (15-20 kg) were anesthetized with 30 mg/kg of intravenous sodium pentobarbital and ventilated according to the nomogram of Kleiman and Radford (13). The femoral artery was catheterized for continuous blood pressure monitoring and to collect blood samples; while the femoral vein was cannulated for infusion of creatinine (20 mg/min) to measure GFR, and additional anesthesia, as well as to infuse XL-Hb or dextran. Through a left flank incision transonic flow probes (Transonic Systems, Inc., New York, USA) were placed in the mesenteric, renal and iliac segments proximal to the aorta for continuous blood flow monitoring. A curved 23-

gauge needle was inserted into each of these arteries at the distal segment to avoid interferences with flow measurement. The needles were connected via PE 50 tubing to injection ports attached to syringe pumps. Saline was continuously infused, 0.5 ml/min, into each vascular bed. Bolus injections of two doses of arachidonic acid (AA) (205 nM and 410 nM in the iliac and 410 nM and 820 nM in the renal and mesenteric arteries) were injected into each vascular bed before and one hour after volume expansion to detect possible changes in vascular reactivity due to enhanced prostaglandin formation produced by the catalytic actions of XL-Hb. The left ureter was also cannulated to collect urine samples.

Before XL-Hb or dextran infusions were started, averaged values from two 20 min. periods were considered for basal situation (periods 1 and 2). The infusion of either 6% Dextran or XL-Hb (10% blood volume) was given by continuous infusion over 20 minutes. Thereafter, three 20 min. periods (3,4, and 5) were considered to evaluate the effects of the two substances. Urine samples were collected during each clearance period to measure urine flow, total and fractional Na^+ excretion rates, osmolality and creatinine levels. Blood samples for measuring plasma creatinine and hematocrit levels were collected at the midpoint of each clearance period, while samples to measure plasma renin activity (PRA) and atrial natriuretic peptide (ANP) were obtained at the end of the first control period and 40 minutes after the infusion (end of period 4).

Plasma and urine creatinine were measured using a Beckman Creatinine Analyzer, and creatinine clearance was used to estimate GFR. Osmolality was measured by a freezing point depression osmometer (Precision System 5004); Na^+ concentration was measured using a flame photometer (Instrumentation Laboratory IL943). Finally PRA and ANP were measured by commercial radioimmunoassay Kits (DuPont NEA-105 and Peninsula RIK-8798, respectively).

In vitro effects of XL-Hb in isolated renal arteries

The experiments were performed on rings (3-5 mm in length) of renal arteries taken from dogs (15-20 kg) anesthetized with sodium pentobarbital (30 mg/kg iv) and euthanized via exanguination. The arteries were placed in modified Krebs-Ringer bicarbonate solution [control solution (in mM): 118.3 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25.0 $NaHCO_3$, 0.026 calcium EDTA,

and 11.1 glucose]. Each ring was connected to an isometric force transducer (Gould UTC-2, Oxnard, CA, USA) and suspended in an organ chamber filled with 25 ml of control solution (37°C, pH 7.4) and gassed with 94% O_2-6% CO_2 . Isometric tension was recorded continuously.

Each ring was gradually stretched to the optimal point of its length-tension curve as determined by the contractions to norepinephrine (3×10^{-7} M) (13). Optimal resting tensions were 10 g for renal arteries (12). The functional integrity of endothelium was tested by the presence of relaxations to acetylcholine (10^{-6}M).

The following pharmacological agents were used: acetylcholine hydrochloride (Sigma, St. Louis, MO), calcium ionophore A23187 (Sigma), L-norepinephrine (Sigma) and papaverine hydrochloride (Sigma). Stock solutions of the drugs were prepared fresh every day. Drugs were dissolved in distilled water such that volumes of <0.2 ml were added to the organ chambers. All concentrations are expressed as final molar (M) concentration in the bath solution.

Cross-linked hemoglobin was obtained from Walter Reed Army Institute of Research (Washington D.C., USA). The solution was prepared from stroma-free human hemoglobin from outdated blood modified with bis (3,5-dibromosalicyl) fumarate according to the method of Snyder (21). The cross-linked hemoglobin was formulated in Ringer acetate (7 g/100 ml) and maintained at 4°C until the day of use. At that time it was passed through a 0.22 μm filter to remove particulate matter, then warmed to 37°C by placing the bag in a water bath. The incubation time for XL-Hb was 30 min.

Concentration-response curves were obtained in a cumulative fashion. Several rings cut from the same artery were studied in parallel; only one concentration-response curve was made per preparation. The relaxations were expressed as a percentage of maximal relaxations to papaverine (3×10^{-4}M).

Statistical analysis.

The results are expressed as means ± SEM. Results from the two control periods were averaged and compared to each of the post infusion periods with a randomized block analysis of variance. When the F value yielded a $p<0.05$, difference between clearances were determined by

Newman-Keuls multiple range test. Differences between Dextran and XL-Hb infusions were evaluated using an unpaired Student's t-test. With respect to the *in vitro* studies, n refers to the number of dogs and the statistical evaluation of the data was performed by Student's t-test for paired observations. A $p<0.05$ was considered significant.

RESULTS

Infusion of XL-Hb

Infusion of XL-Hb induced a 13.5% decrease in hematocrit levels (from 39.5±2.06% to 34.17±1.23%, $p<0.01$). This infusion (control value of periods 1 and 2 vs. averaged increments in periods 3-5) produced significant and sustained decreases in mesenteric (210±27 to 147±22 ml/min, $p<0.05$), renal (198±14 to 134±12 ml/min, $p<0.05$), and iliac (135±17 to 82±10 ml/min, $p<0.05$) blood flows (Fig. 1b-d) while mean arterial pressure increased significantly from 114±4 to 147±10 mm Hg ($p<0.05$) (Fig. 1a). GFR (Fig. 2a) remained unchanged, as well as total urinary and fractional sodium excretion (Fig. 2b-c). Urinary flow during XL-Hb administration increased by 81±42.4%. In addition, XL-Hb administration resulted in a 75% decrease in plasma renin activity and a 168% increase in atrial natriuretic peptide levels (Table 1).

Finally, in the three vascular beds the two doses of AA systematically increased blood flow after the infusion of XL-Hb (Fig. 3a-c), but not in the basal period.

Infusion of Dextran

Dextran infusion induced a decrease of 13.8 % of hematocrit levels (from 36.17 ± 1.5% to 31.17 ± 1.67%, $p< 0.01$). In contrast with the effects of XL-Hb, dextran infusion produced significant increases in mesenteric (346±43 to 391±34 ml/min, $p<0.05$), renal (182±23 to 211 ±27 ml/min, $p<0.05$), and a transient increase in iliac (176±23 to 186±17 ml/min) blood flows (Fig. 1b-d), without concomitant changes in mean arterial blood pressure (Fig. 1a). Sodium excretion and fractional sodium excretion (Fig. 2b-c) rates significantly increased from 51±17 to 168±44 μEq/min,

($p<0.05$) ml/min ($p<0.05$) and from 0.96±.32 to 2.78±0.96% ($p<0.05$), respectively, without any change in GFR (Fig. 2a). The lack of changes in GFR associated to the significant increments in sodium excretion resulted in a significant elevation of the calculated FeNa which were comparable to the increments seen for total sodium excretion (2c). Urinary flow increased by 206.8±43.9% ($p=0.066$ with respect to the increase observed in XL-Hb group). In addition, Dextran infusion resulted in a 47% decrease in plasma renin activity and a 16% increase in atrial natriuretic peptide levels (Table 1).

Intraarterial bolus injections of AA (Fig. 3a-c) did not alter blood flow in any vascular bed before or after Dextran infusion.

Effects of XL-Hb on renal artery relaxation *in vitro* induced by A23187 and acetylcholine

It can be seen in Figure 4 that under control conditions exposure of renal arteries to concentrations of A23187 of 8, 7.5, and 7 (-logM) evoke a relaxation of 20%, 66%, and 95%, respectively. The vasodilator effect was significantly blunted by the administration of XL-Hb (10^{-6} M), since the administration of the first two doses of A23187 (8 and 7.5, -logM) failed to produce a change in the basal tone, whereas the concentration of -7 logM evoked only a 50% relaxation of the arterial strips. This represents a 50% decrease with respect to the relaxation evoked by the same dose of A23187 in the absence of XL-Hb. Figure 5 shows that the inhibitory effects of XL-Hb were also exerted during the relaxation induced by acetylcholine (Ach). In fact, the 33%, 60% and 81% relaxation induced by -7.5, -7 and -6.5 (logM) of Ach were almost completely abolished in the presence of XL-Hb, while the 88% relaxation induced by -6 logM of Ach was reduced to 44%.

DISCUSSION

Since Dextran (MW 55,300) and XL-Hb (MW 64,000) have high molecular weights and both solutions were matched for osmolality and sodium content, it would be reasonable to assume that the magnitude of both volume infusions was comparable. In fact, the average fall of hematocrit in both

groups of dogs, 13.8% and 13.5% respectively, was similar. In spite of these similarities, the consequences derived from the intravenous infusion of both substances were markedly different. The acute infusion of XL-Hb was followed by an increase in MAP which was accompanied by peripheral vasoconstriction in several vascular beds. In fact, the estimated blood flows in renal, mesenteric, and iliac vasculatures were uniformly decreased by 32, 30, and 39%, respectively. The increase in intrarenal resistance seen during the XL-Hb infusion was equally distributed between glomerular afferent and efferent vasculature as GFR did not change. Under these conditions, urinary volume, and total and fractional excretion of sodium remained within the range of values recorded in the control periods, despite the volume expansion induced by the infusion and the increase in blood pressure. This fact indicates that the increase in systemic blood pressure due to the administration of XL-Hb failed to produce pressure-induced natriuresis. An important issue disclosed by our results shows that the hemodynamic and renal effects produced by XL-Hb differs from those produced by a neutral volume expander of approximately the same molecular weight, such as dextran.

It has been previously reported that the intravenous administration of Dextran produces a significant increase in cardiac output which fails to increase mean arterial pressure because of a compensatory reduction in total peripheral resistance (2). These results are in agreement with our findings which show that Dextran infusion did not modify blood pressure levels, but increased transiently the iliac and sustainedly the mesenteric and renal blood flows.

The increase in RBF produced by Dextran was not accompanied by changes in GFR, which suggests that the renal vasodilatation affected similarly both glomerular afferent and efferent arterioles in such a manner that glomerular capillary pressure remained fairly constant. However, urine flow and total and fractional Na^+ excretion were significantly increased, thus indicating that the major cause for the observed natriuresis consisted of a reduction of tubular sodium reabsorption (23). A decrease in tubular reabsortion under these conditions has been attributed to changes in glomerular-tubular balance, to a decrease in PRA, as well as to a withdrawal of the renal sympathetic activity (2,23). Furthermore, there is numerous evidence pointing out that the volume expansion-induced natriuresis is very significantly mediated by the elevation of ANP (6) and by the stimulation of NO

synthesis (17). Our results are also in agreement with some of these previous observations since Dextran infusion was attended by a significant fall in PRA and by a marked elevation in the circulating levels of ANP.

It has been reported that XL-Hb exerts an effective scavenging action on circulating NO, as this molecule possesses a high affinity for the heme groups (9,10). The scavenging of NO could account for the rise in blood pressure and the decrease in the three regional blood flows, as well as the blunted natriuresis, that we found in our study. This statement is supported by comparable results which were observed when L-NAME, a potent inhibitor of NO synthesis, was infused into rats (14) and dogs (18-19). The response in these animals involves the elevation of MAP without a proportional increase in sodium excretion because of the counteracting antinatriuretic effect of NO suppression (17). Additional support to the idea that the vasoconstrictor effect of XL-Hb seen in our *in vivo* study is due to the scavenging effect of NO is provided by our observations *in vitro* in isolated renal arteries. This experiment shows that HL-Hb blunted the vasodilatory response induced by two known endothelium-dependent vasodilators, such as acetylcholine and A23187.

Furthermore, in a previous study conducted by Schultz, et al (20) it was shown that the administration of diaspirin cross-linked hemoglobin (DCL-Hb) produced a significant increase of MAP which after reaching the peak was significantly reduced by the intravenous infusion of NO donors (such as nitroglycerine, NTG) or NO synthesis precursors (such as L-arginine). In the absence of appropriate controls these results are difficult to interpret because the vasodilator effect of NO donors could reduce any kind of hypertension. On the other hand, the hypotensive effect of L-argenine may be indicating that this amino acid is capable of increasing the producion of NO to a point that overrides the scavenging effect of DCL-Hb or that the vasoconstrictor effects of DCL-Hb are not due to the binding of NO by the HEM group. The authors favored the first possibility because they show that the inactivation of the HEM group by conversion to cyanomethemoglobin fail to induce hypertension. The concept that NO in any form would react with oxygenated HEM groups inactivating the NO and leaving a positive charge on the molecules of hemoglobin is at present highly elevated. Jia, et al (11) have recently shown that NO would preferentially react with a thiol (a sulfur

and hydrogen) -group of the two cisteine molecules contained in hemoglobin; while the binding of NO to the HEM group has a lower affinity.

The biological activity of hemoglobin containing NO bound to the thiol groups only; or to the HEM groups only or to both groups was tested by Jia, et al (11) in isolated arteries. It was found that the vessels constricted to all three hemoglobin preparations but the constrictor effect was greater when both the thiol and HEM groups did not contain NO. From our results we cannot determine which chemical group was responsible for binding NO. However, it is conceivable that the continuous uptake of NO by XL-Hb from the lumen of the vessel may create a low concentration gradient of NO which will decrease the diffusion of NO toward the smooth muscle.

The cyclooxygenase-like activity of the heme group has been well characterized *in vitro* (3,4,16,22, 25-26). However, the hypertensive effect that we have achieved during XL-Hb administration does not seem to agree with these *in vitro* findings. In fact, our results support the idea that Hb-dependent stimulation of PG synthesis may be of a rare occurrence under physiological conditions when all hemoglobin is contained in the red cells or even circulating free into the vascular compartment as it was the case of XL-Hb (25). In fact, this cyclooxygenase-like effect of Hb was apparent only after an intravenous bolus infusion of the substrate was given. Furthermore, the predominant effect produced by the infusion of XL-Hb was a generalized vasoconstriction in all vascular beds studied.

An interesting and novel finding of our study was the pronounced elevation of circulating ANP observed during the infusion of XL-Hb. This increase cannot be ascribed to a volume expansion as it was 11-fold higher than the increase induced by a similar volume expansion induced by the infusion of Dextran. Although our study does not allow further speculation of the mechanism by which XL-Hb influenced the concentration of ANP in blood, there are evidences showing that ANP release could be stimulated by changes in the production of humoral factors derived from the endothelial cells (15). In our study, the 2.7-fold higher increase of circulating ANP achieved with XL-Hb expansion (compared to Dextran) was not associated with a proportional increase in Na^+ excretion. This fact suggests that XL-Hb produced a blunted natriuresis despite the higher increase in

ANP levels observed in the XL-Hb group.

In summary, this study demonstrates that the acute infusion of XL-Hb into euvolemic dogs induces a significant vasoconstrictor effect in three major vascular beds (renal, mesenteric, and iliac), leading to an increase in blood pressure, and a blunted natriuresis. These alterations can not be attributed to volume expansion, as they were not observed when a similar expansion was induced with Dextran. Therefore, these differences in the responses may be more related to specific biological actions of XL-Hb, such as an NO scavenging effect. This statement is supported by the fact that NO synthesis inhibition with L-NAME induces comparable effects to those obtained with XL-Hb, as well as the effect of XL-Hb in isolated renal arteries in the present study. Finally, the possible stimulation of vasodilator prostaglandin synthesis during XL-Hb infusion was not observed in our *in vivo* studies, as indicated by the elevation of blood pressure and the reduction in the three arterial blood flows.

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FIGURE LEGENDS

Figure 1a-d. Changes in mean arterial pressure (MAP) and in iliac (IBF), mesenteric (MBF), and renal (RBF) blood flows observed after the i.v. infusion of Dextran (closed circles) or XL-Hb (open circles) during periods 2, 3, 4, and 5. Period 1 served as a baseline.
* $p<0.05$ between the treatment groups and † $p<0.05$ with respect to the basal period

Figure 2a-c. Changes in glomerular filtration rate (GFR), urinary sodium excretion (UNaV) and fractional excretion of sodium (FeNa) during the same conditions explained in the previous figure.
* $p<0.05$ between the treatment groups and † $p<0.05$ with respect to the basal period

Figure 3a-c. Percent (%) increase in iliac (IBF), mesenteric (MBF) and renal (RBF) blood flows induced by the bolus injection of two doses of arachidonic acid (AA) given during the control periods and after infusion of Dextran or XL-Hb (see reference bars).
* $p<0.05$ with respect to basal period

Figure 4. Concentration-response curves to A23187 in canine renal arteries in the absence and presence of XL-Hb. Relaxations were obtained during contractions to norepinephrine (3×10^{-7} M). Data are shown as means±SE and expressed as percent of maximal relaxation induced by papaverine (3×10^{-4} M, n=5 for control rings and in the presence of XL-Hb, respectively).
* $p<0.05$ with respect to control rings.

Figure 5. Concentration-response curves to acetylcholine in canine renal arteries in the absence and presence of XL-Hb. Relaxations were obtained during contractions to norepinephrine (3×10^{-7} M). Data are shown as means±SE and expressed as percent of maximal relaxation induced by papaverine (3×10^{-4} M, n=5 for control rings and in the presence of XL-Hb, respectively). * $p<0.05$ with respect to control rings.

Table 1. Hormonal values obtained before and after infusion of dextran or XL-Hb

	Dextran Control	Dextran Infusion	XL-Hb Control	XL-Hb Infusion
PRA (ng AI/ml/hr)	3.4±1.6	1.8 ±0.5	5.6 ±0.8	1.4 ±0.6*
ANP (pg/ml)	72 ± 9	83 ±12*	103 ± 13	276±34*

Mean ±SEM. * $p<0.05$ vs. basal period. PRA: plasma renin activity. ANP: atrial natriuretic peptide.

PERSPECTIVES

The therapeutic efficacy of blood transfusion has been hampered by the existence of transmissible disease such as AIDS and by accidents linked to blood storage. These problems could now be solved by using stroma-free solutions of newly polymerized hemoglobin (this component has been cross-linked hemoglobin) XL-Hb. The study shows that the I.V. infusion of XL-Hb differs from the effect produced by other volume expanders, such as Dextran, because it induces a marked increase in peripheral vascular resistance (such as renal mesenteric and iliac vasculatures) with a marked elevation of mean arterial pressure. These changes, however, are not accompanied by any alteration in sodium excretion. This hypertensive an anti-natriuretic effects are most likely produced by a reduction in the concentration of NO which is bound to hemoglobin. These actions will have to be taken in consideration if cross-linked hemoglobin is used as a volume expander in hypovolemic conditions in humans.

APPENDIX

PROJECT 2

"Effects of Xl-Hgb Solutions on Catecholamine Release from the Adrenal Medulla and Vascular Sympathetic Nerve Terminals"

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NOREPINEPHRINE RELEASE DURING VASOCONSTRICTION INDUCED BY CROSS-LINKED HEMOGLOBIN

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Summary

The pressor effect of hemoglobin-based blood substitutes is due partly to their capacity to scavenge nitric oxide (NO), a potent vasodilator. NO also appears to modulate the release of norepinephrine (NE) from sympathetic nerve endings in some blood vessels. Thus studies were designed to determine if contraction occurring in response to $\alpha\alpha$-cross-linked hemoglobin (XL-Hb) is due in part to increased exit of NE from vascular nerve endings. Helical strips of canine femoral artery were superfused *in vitro* with Krebs-Ringer solution and, for each strip, the overflow of NE into the superfusate as well as contractile responses were measured concurrently during basal conditions, during nerve stimulation and during tyramine-evoked release of NE. XL-Hb (10 μM) contracted unstimulated strips without affecting NE overflow. NE overflow also was unchanged by N^G-monomethyl-L-arginine (L-NMMA; 300 μM), an inhibitor of NO synthesis; by sodium nitroprusside (SNP; 1 μM) an NO donor; by a combination of XL-Hb and L-NMMA; or of XL-Hb and SNP. These treatments contracted the strips to the same degree as did XL-Hb alone, except for SNP, which induced relaxation. Transmural stimulation of the strips at 2 and 10 Hz induced NE overflow and contraction, neither of which was affected by any treatment except SNP which significantly ($P < 0.05$) increased NE overflow while inhibiting contraction. In other experiments, XL-Hb augmented contractions induced by tyramine (10 μM) although the resulting NE release was unaffected. These results suggest that, in the femoral artery, contractions induced by XL-Hb are not due to increased efflux of NE from vascular nerve endings but are consistent with inhibition of the the actions of NO.

Key Words: cross-linked hemoglobin, norepinephrine, nitric oxide, blood pressure, 3,4-dihydroxyphenylglycol, femoral artery

The need for an oxygen-carrying blood substitute for use following trauma has long been recognized (1). In recent years the threat of transmitting pathogenic viruses during transfusions has intensified the search. One substitute is based upon hemoglobin which has

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been interdimerically cross-linked with bis (3,5-dibromosalicyl) fumarate between α chains ($\alpha\alpha$-cross-linked hemoglobin; XL-Hb). Other terminology has been used to describe the same compound, i.e., $\alpha\alpha$Hb (2), DCLHb (3), and HbXL99α (4). This hemoglobin derivitive is an effective oxygen carrier and resuscitative fluid (2), however when administered, it often causes hypertension (3,5). The reasons for the pressor effect are not fully understood, although they appear to be mediated by mechanisms not directly involving the central nervous system (3). XL-Hb, like native hemoglobin, has a high affinity for nitric oxide (NO), and recent studies have shown that the increase in arterial pressure induced by XL-Hb is associated with its propensity to scavenge this vasodilator (6).

In blood vessels, NO is produced predominantly within the endothelium, with the primary vascular target being soluble guanylate cyclase within smooth muscle cells (7). The neurotransmitter norepinephrine (NE) is stored within vascular sympathetic nerve endings and is released exocytotically into the neuroeffector junction, resulting in a contractile response that opposes the actions of NO. Interestingly, the exocytotic release of NE appears to be modulated by NO in some blood vessels (8-10). Thus we hypothesized that the pressor effect of XL-Hb might be due partly to its binding to NO, thereby reducing the amount of NO available to signal a change in NE release. As a result the release of NE into the neuroeffector junction could be increased. Alternatively XL-Hb might act directly through some as yet unknown mechanism affecting the neuronal membrane. The aim of this study was to determine if the vasoconstriction induced by XL-Hb is associated with increased efflux of NE from sympathetic nerve endings.

The canine femoral artery contracts robustly when exposed to hemoglobin. In *in vitro* experiments using helical strips of the vessel, XL-Hb was applied during basal conditions, and the efflux of NE (whether by exocytosis, carrier-mediated release or diffusion) from sympathetic nerve endings during the ensuing contractions was quantified. Also, the release of NE was induced by nerve depolarization as well as by tyramine, a sympathomimetic amine, and the effects of XL-Hb on these differing release processes as well as on the concomitant contractions of the vessels were quantified.

Methods

Tissue preparation: These studies were approved by the Institutional Animal Care and Use Committee. Tissues were removed simultaneously from adult mongrel dogs for studies in this and seven other research laboratories that make up the Mayo program of shared-use of animals in research. Dogs of either sex were first anesthetized with pentobarbital sodium (30 mg/kg); for this study both femoral arteries were removed and placed in oxygenated Krebs-Ringer solution (K-R; 11). The removed vessels were dissected free of perivascular tissue and each was cut carefully into a helical strip approximately 0.5 x 6 cm. Each strip was then cut longitudinally to yield two strips of equal size; thus four vessel strips were studied from each dog.

Superfusion procedure: The strips were superfused at 37°C as described previously (12). Briefly, each strip was suspended vertically in a 1 x 10 cm glass tissue chamber. The bottom end of each strip was anchored, and the top end was tied by a thread to a force-displacement transducer. K-R which was held in a reservoir and aerated with 20% O_2, 5% CO_2, 75% N_2 was pumped at 2 ml/min to the top of each chamber where it then dripped over the enclosed strip. The contractile activity of each strip was measured continuously using a strip-chart

recorder. In some instances transmural stimulation (TMS; 10 V, 0.2 msec duration, 2 or 10 Hz) was applied via two parallel platinum wire electrodes in contact with each strip throughout its length and wired to an electrical stimulator.

The vessel strips, after mounting, were equilibrated for 30 min, then the K-R was changed to K-R containing desmethylimiprimine (DMI; 1 μM), corticosterone (40 μM), and indomethacin (10 μM) in order to inhibit neuronal and extraneuronal NE uptakes and prostaglandin synthesis, respectively (13-15). After a further 30 min, each strip was stretched manually in small increments until 6 g passive force was applied. Preliminary experiments determined this to be the average optimum point of the length-tension curve for femoral artery strips in this experimental model. The vessels were rested for 90 min, then collection of superfusate was begun.

Collections were made during three 25-minute periods, each 60 min apart. For each of these three periods, collections were done in 5-min intervals, with TMS being applied only during the second 5-min interval of each period. Hence for each period, superfusate collection was initiated during basal conditions, followed by collection during TMS, and finally during three subsequent post-TMS intervals. TMS was at 2 Hz for the first and second set of collections and at 10 Hz for the third set. In control vessels, K-R (containing DMI, corticosterone and indomethacin) was applied throughout. In other vessels, K-R containing these inhibitors and either XL-Hb (10 μM), N^G-methyl-L-arginine (L-NMMA; 300 μM), sodium nitroprusside (SNP; 1 μM), L-NMMA and XL-Hb together, or SNP and XL-Hb together was applied 20 min before the second set of collections and continued throughout. After the 15 collection intervals were completed, the strips were removed from the chambers, then blotted dry, weighed, and stored at -80°C. In all vessel strips studied, the functional integrity of the endothelium was assessed before collection of superfusate by establishing relaxation to acetylcholine (1 μM) during contraction induced by 4 Hz TMS.

Basal efflux of NE: The amount of NE which overflowed from each vessel strip into the superfusate during each 5-min interval was quantified (see below). To compare the effect of each treatment on basal efflux of NE, the amount of NE which overflowed during the basal interval of the second set of collections (treatment added) was expressed as the percentage of the amount which overflowed during the basal interval of the first set of collections (prior to treatment). In each strip, the contractile effect of XL-Hb or of the other treatments on basal tone was measured concurrently with NE overflow, and was expressed as the percentage of the contraction elicited by the first 2 Hz TMS.

Exocytotic release of NE: For each set of superfusate collections, the amounts of NE above basal levels which overflowed during the interval of TMS and during the three post-stimulation intervals were summated and considered to represent the amount of NE released exocytotically by that stimulation, since neuronal and extraneuronal uptakes of NE had been inhibited. The first set of collections was done under identical conditions in all experiments: using 2 Hz TMS, and before addition of XL-Hb or drugs to the K-R superfusing medium. Therefore for comparative purposes, the amount of NE released as well as the maximum contraction attained during each of the two subsequent sets of collections were expressed as the percentage of these respective values that were measured during this first period. Application of XL-Hb or of the other compounds during basal tonus of the vessel strips caused contraction or relaxation, and these responses were taken into account in measuring the contractions attributed to TMS; for this purpose contraction at the time immediately before TMS began was considered to be zero.

Tyramine-evoked release of NE: A second protocol, similar to that described above, up to the point of the second set of collections, was designed to determine the effects of XL-Hb on the carrier-mediated release of NE. In these experiments TMS was not applied during the second set of collections; instead K-R containing tyramine (10 μM) was applied, during intervals two and three, and the experiments were terminated after this second set of collections. Paired vessel strips were superfused simultaneously; one strip of each pair was not exposed to XL-Hb and served as the control; XL-Hb (10 μM) was added to the K-R of the other strip 20 min before the second set of collections and remained throughout. DMI was omitted in these experiments so that the neuronal amine uptake mechanism remained functional. The NE in each sample of superfusate was quantified as described. 3,4-Dihydroxyphenylglycol (DHPG), the major intraneuronal metabolite of NE diffused from nerve endings into the superfusate concurrently with released NE and, in these experiments, was assayed in the same samples by using the method used for NE. The contractile activity of each strip was measured continuously during each set of collections.

Separation and measurement of NE and DHPG: The NE (and in the tyramine experiments, the DHPG also) which overflowed into the superfusate during each collection interval was adsorbed onto a Sep-Pak Plus C-18 cartridge (Waters, Milford MA, USA) attached directly to the bottom of the superfusion chamber and was quantified by reversed-phase HPLC with coulometric detection (12,16). The concentration of analytes in superfusate was expressed as pmol or pmol/min and adjusted to 100 mg tissue weight, the average weight being 71.4$\pm$ 1.4 mg (N = 53). The amounts of NE and DHPG measured in each superfusate sample were adjusted for recovery. Of the K-R additions, only XL-Hb significantly affected NE recovery, which was 83.3 $\pm$ 1.7% (N = 25), as compared to 95.4 $\pm$ 0.7% (N = 28) in samples without XL-Hb. Similarly, DHPG recovery was 75.9 $\pm$ 7.0% and 65.1 $\pm$ 6.6% (N = 8 each) in control and in XL-Hb-treated samples, respectively. The limits of detection of NE and of DHPG for the assay, expressed per 100 mg of artery, were 7 fmol/min and 8 fmol/min, respectively.

Preparation of XL-Hb: XL-Hb was obtained from the U.S. Army Medical Research and Development Command. It was prepared from stroma-free hemoglobin from outdated human blood and was modified with bis (3,5-dibromosalicyl) fumarate according to the method of Snyder et al (4). The solution was formulated in Ringer acetate (7 g/100 ml), and stored at -80°C. Prior to use, it was gradually warmed and filtered through a 0.2 μm filter.

Statistics: Data are presented as mean $\pm$ SEM. For experiments examining basal and TMS-induced NE release, the Student's t-test was used to determine if any of the treatments caused differences in NE overflow or in contraction from those measured in the control vessel strips. In the experiments examining tyramine-induced NE release, differences over time in the overflows of NE and of DHPG as well as in contractions resulting from application of XL-Hb were determined using two-way ANOVA with repeated-measures. Differences were considered significant at $P < 0.05$.

Results

Basal efflux of NE: In all experiments, treatments were added after the first set of collections, therefore the amount of NE which overflowed during the basal interval of the first set of collections was used as a basis for comparing the amounts which overflowed during the basal interval of the second set of collections (Figure 1A). Small amounts of NE were

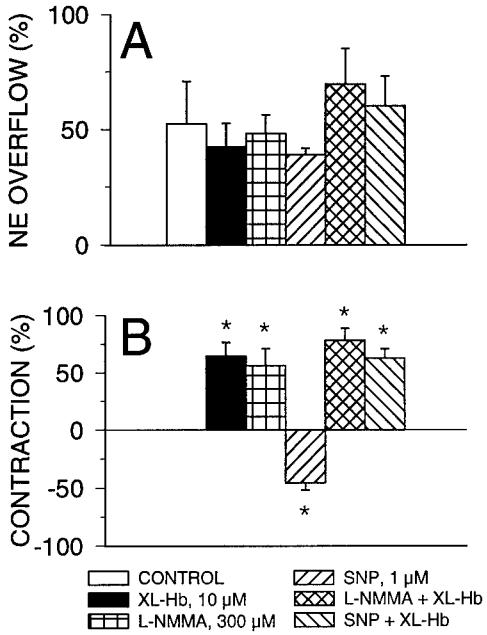

Fig. 1

NE overflow (A) and contraction (B) during basal conditions in femoral artery strips superfused *in vitro*; effects of XL-Hb and of various treatments which modify tissue levels of NO. NE overflow was measured during a 5-min period and is expressed as the percentage of the NE which overflowed during a comparable 5-min period prior to treatment. Contraction is expressed as the percentage of the contraction induced by stimulation of the vessel at 2 Hz prior to treatment. Values are means ± SEM of determinations from five different experiments. *, Significant difference from corresponding value in control vessel, $P < 0.05$.

measured in the basal superfusate of the control arteries (0.34 ± 0.07 pmol/5 min). The amount decreased over time, with only about 50% as much being present during the second basal interval as during the first (Figure 1A). The amounts of NE in the superfusate during basal conditions were unchanged from control amounts by XL-Hb or by any of the other treatments (Figure 1A). During basal conditions the tone of the control vessel strips was unchanged, however XL-Hb contracted other strips significantly (Figure 1B). L-NMMA contracted unstimulated strips to the same degree as did XL-Hb, whereas SNP caused a relaxation that was significant. XL-Hb had no additional effect on the contraction induced by L-NMMA, whereas the relaxation induced by SNP was reversed by XL-Hb.

Exocytotic release of NE: Stimulation of the control femoral arterial strips by TMS for 5 min caused release of substantial amounts of NE (11.1 ± 1.4 total pmol at 2 Hz and 34.0 ± 4.4 total pmol at 10 Hz). Only SNP significantly increased NE release at both frequencies tested compared to controls; other treatments were without effect (Figures 2A and 2B). In the control vessel strips, the maximal TMS-induced contractions reached 2.5 ± 0.3 g at 2 Hz and 3.8 ± 0.4 g at 10 Hz (N = 5 each). Vessel strips exposed to SNP alone did not contract to 2 Hz and contracted weakly to 10 Hz whereas the other treatments were without effect on contractions (Figures 2C and 2D).

Tyramine-evoked release of NE: In other experiments, NE release from femoral artery strips was induced by exposure to tyramine (10 µM) for 10 min. In strips not exposed to XL-Hb, tyramine induced the release of NE in amounts that were about one-half of those elicited by 2 Hz TMS, and these amounts were unchanged in strips treated with XL-Hb (Figures 3A and 3B). In control vessel strips, the rate of DHPG overflow was about double that of NE. XL-Hb was without effect on the overflows of NE and DHPG, both during basal conditions and during exposure to tyramine (Figure 3C and 3D). The contractions to tyramine in the control

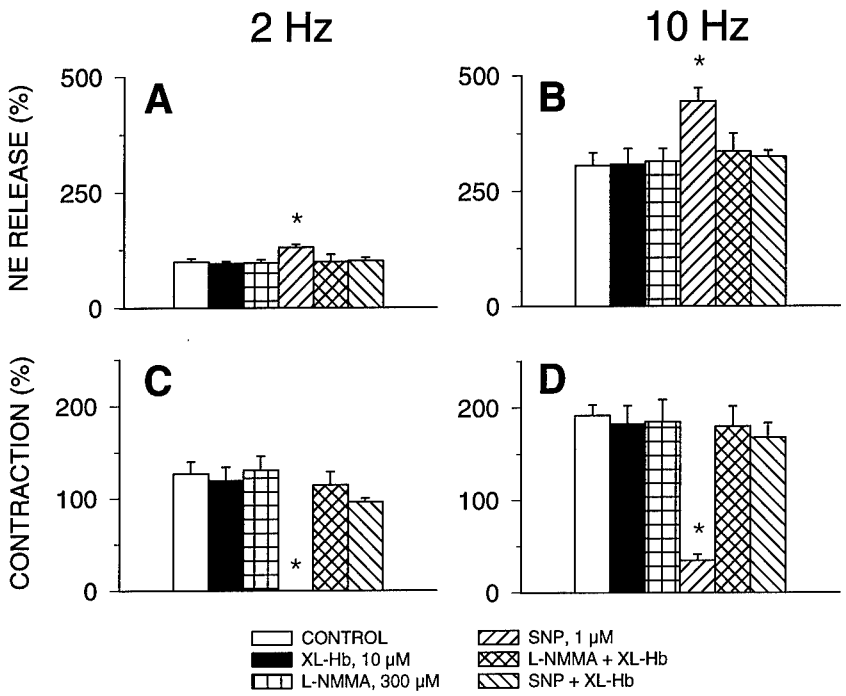

Fig. 2

NE overflow (A,B) and maximum contraction (C,D) induced by stimulation for 5 min at 2 Hz or 10 Hz in femoral artery strips superfused *in vitro*; effects of XL-Hb and of various treatments which modify tissue levels of NO. NE overflow and maximum contraction are expressed as the percentage of these respective values induced by 5 min stimulation at 2 Hz prior to treatment. Values are means ± SEM of determinations from five different experiments. *, Significant difference from corresponding value in control vessel, $P < 0.05$.

strips were about half the magnitude of those elicited by 2 Hz stimulation. Further, tyramine-induced contractions in the XL-Hb-treated strips were significantly higher than those induced by tyramine in the control strips, even though, in both groups, the *pattern* of contraction over time was similar and corresponded to the concurrent pattern of NE release (Figures 3A-3F).

Discussion

Basal efflux of NE: XL-Hb increased the basal tone of strips of femoral artery, causing contractions of a magnitude similar to those elicited by nerve depolarization at low frequencies. However this XL-Hb-induced contraction was not due to increased efflux of NE from vascular nerve endings. Endogenous NO, at least at the levels produced in these experiments, did not modulate the efflux of NE during basal conditions since basal levels of NE in superfusate were unchanged by L-NMMA, an inhibitor of NO synthesis. Additionally no mechanism appears to be in place for NO, when present in added amounts, to modulate the basal efflux of NE, since SNP was without effect on NE levels in superfusate.

The XL-Hb-induced contraction of the femoral artery strips during basal conditions was

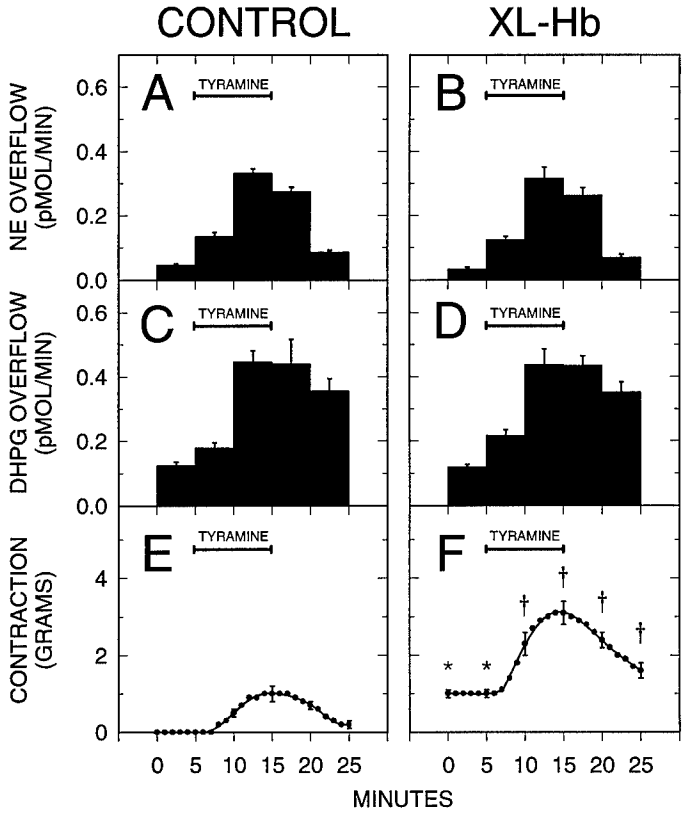

Fig. 3

Overflows of NE (A,B) and DHPG (C,D) as well as contractions (E,F) induced by tyramine (10 μM) in femoral artery strips superfused *in vitro*; effect of XL-Hb (10 μM). Superfusate was collected in five consecutive 5-min intervals, with tyramine being applied during intervals 2 and 3 as indicated by the bars. Values are means $\pm$ SEM of seven determinations. XL-Hb induced significant ($P < 0.05$) contractions during basal conditions (*, Students t-test) and augmented contractions to tyramine (†, two-way ANOVA with repeated measures).

consistent with reduced levels of NO in the biophase. Inhibition of NO synthesis by L-NMMA contracted the vessels to the same degree as did XL-Hb. Also, XL-Hb reversed the relaxation by SNP, an agent with an action that derives primarily from increased tissue levels of NO. Additionally, contractions induced by XL-Hb and L-NMMA combined showed no additive effects suggesting that the effects of XL-Hb in the femoral artery are mediated through the same pathway as are those of L-NMMA, namely through NO (17).

It appears likely that the NE which was measured in the superfusate during basal conditions exited the sympathetic nerve endings by diffusion. Although NE is highly polar at physiological pH, it diffuses across the neuronal membrane in small amounts (18). Release of NE during unstimulated conditions in this vessel is Ca^{++}-independent (data not shown), further reducing the possibility of exocytosis. Carrier-mediated NE release during basal

conditions was also unlikely because, in these experiments, the neuronal amine uptake carrier had been inhibited by DMI; also the ATP-driven Na^+ gradient across the neuronal membrane favors inward but not outward transport of NE. Therefore the present results strongly suggest that XL-Hb has no effect on diffusion of NE across the neuronal membrane.

Exocytotic release of NE: Under the experimental conditions of the present study, the amount of NE which overflowed into the superfusate as a result of nerve depolarization by TMS was equated with the amount which was released exocytotically since neuronal and extraneuronal amine uptakes were inhibited. The experiments establish that neither XL-Hb nor NO significantly affect the exocytotic release of NE or the resultant contractions in the femoral artery, whether induced by low (2 Hz) or high (10 Hz) physiological frequencies of nerve depolarization. The data also indicate, although indirectly, that XL-Hb does not affect exocytotic NE release through a mechanism independent of NO (e.g., through interaction with endothelin; 19), since release was the same in vessels exposed to XL-Hb either in the presence or absence of L-NMMA.

SNP increased NE release at both frequencies of TMS tested indicating that, although within the parameters of these experiments, endogenous NO was without effect on NE release, the mechanisms are present by which NO could augment NE release when NO levels are sufficiently high. This raises an interesting possibility regarding the interaction of NE and NO in maintaining vascular homeostasis *in vivo*. Increased plasma levels of NO, due largely to its synthesis via the inducible isoform of NO synthase, are characteristic of several pathological conditions (20). For example, septic shock induces elevated plasma levels of both NO and NE (21,22). Therefore it is possible that endogenous NO, at levels sufficient to induce hypotension could trigger increased exocytosis of NE, which would serve as a physiological braking mechanism to partially counteract the detrimental effects of overproduction of NO.

Our finding that NO, at levels above those produced endogenously, increased NE release are not in agreement with a previous study on isolated canine mesenteric arteries in which a decrease in the release of NE induced by SNP and other NO donors was reported (8). The reasons for this discrepancy may relate to the different artery studied and/or to differences in experimental preparations. Endogenous NE was not quantified in those studies, rather the overflow of radioactivity was measured after preloading of vessel strips with 2-[^{14}C]-NE. In addition, the TMS applied was far stronger (e.g., 4 Hz, 2 msec for 10 min); either 40-fold greater (at 2 Hz) or 8-fold greater (at 10 Hz) than the total stimulation used in the present study (which resulted in vigorous contractions).

Tyramine-induced release of NE: Tyramine, a sympathomimetic amine, induces NE release by displacing NE from storage vesicles; the NE subsequently exits the nerve ending via carrier-mediated outward transport (23). That the tyramine-induced increase in NE in superfusate was unchanged by XL-Hb indicates that XL-Hb has no effect on this mechanism of NE release in femoral artery. Equally important in drawing this conclusion is the finding that the levels of DHPG in superfusate were also unchanged by XL-Hb. This lipophyllic metabolite of NE is formed within the neuron by the action of monoamine oxidase (MAO) and the amounts of DHPG measured in superfusate serve as a good indicator of the NE levels within the neuroplasm (24). Thus the neuroplasmic NE levels were probably similar in control vessel strips and in strips exposed to XL-Hb. MAO is strikingly oxygen-sensitive, therefore the lack of effect of XL-Hb on DHPG and NE levels in superfusate also indicates that tissue oxygenation was unchanged in the vessel strips that were exposed to XL-Hb. Thus these data suggest that XL-Hb does not affect carrier-mediated release of NE, the vesicular amine carrier,

or the activity of neuronal MAO.

The potentiation by XL-Hb of tyramine-induced contractions was not due to increased release of NE. The endothelium of the femoral artery contains α_2 adrenoceptors which, when activated, induce the release of NO at levels above those produced during basal conditions (15, 25). The NE which was released by the tyramine probably activated these receptors as well as those on the smooth muscle which caused contraction. Thus the potentiation by XL-Hb of the contractions may be explained by its binding to this released NO. The potentiating effect of XL-Hb may have been present also in the vessel strips which were stimulated with TMS, but was masked by the complicating influences of other vasoactive substances which may have been released by the stimulation. For example, the cotransmitter neuropeptide Y, a highly potent vasoactive compound, is released by transmural stimulation of vascular nerve endings (12, 26), but unlike NE, is not released by tyramine (26).

A further aspect of this study deserves comment. As noted, XL-Hb has a pressor effect when administered to humans or animals *in vivo*. Since hypertension is commonly ascribed to contraction of the resistance vessels, the small arteries and arterioles, the physiological relevance of examining the femoral artery, a conduit vessel, to elucidate the effects of XL-Hb may be questioned. However, the femoral artery contracts vigorously when exposed to XL-Hb, suggesting its appropriateness for this type of study.

In conclusion, this study provides physiological evidence demonstrating that the XL-Hb induced contraction of the femoral artery is not due to increased efflux of NE from vascular nerve endings, whether by exocytosis, carrier-mediated release or by diffusion across the neuronal membrane. Rather the contraction is consistent with inhibition of the actions of NO.

Acknowledgements

This work was supported in part by U.S. Army Contract DAMD 17-93-C-3116-PZ, and by NIH Grant GM 41797. The authors thank Mr. Larry Ward and Mr. Kevin Frank for technical assistance, and Ms. Sharon Heppelmann for secretarial assistance.

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ABSTRACT FORM

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This is a copy of an abstract that was faxed to Dr. Phillips on March 29, 1995.

A EFFECTS OF NITRIC OXIDE ON OVERFLOW OF CATECHOLAMINES FROM PERFUSED DOG ADRENAL GLAND

B Gertrude M. Tyce, Lawrence E. Ward, Larry W. Hunter, and Duane K. Rorie, Departments of Physiology and Anesthesiology, Mayo Clinic/Foundation, Rochester, Minnesota, USA

C Epinephrine (E), norepinephrine (NE) and dopamine (DA) overflow spontaneously from isolated perfused dog adrenal glands. Previously we have shown that approximately 25% of this spontaneous overflow is Ca^{2+} dependent, but is independent of cholinergic, serotonergic or dopaminergic stimulation. The present study was done to determine the actions of nitric oxide (NO) on spontaneous overflow of catecholamines from dog adrenals. Mongrel dogs were anesthetized with 30 mg/kg i.v. sodium pentobarbital and adrenal glands were removed. Krebs-Ringer solution (K-R) was retrogradely perfused at 1.5 ml/min into the adrenolumbar vein and allowed to exit through slits at the ends of the adrenal lobes. After an initial stabilization period a basal sample of perfusate was collected. Perfusion was then continued with K-R containing (a) N^{ω}-monomethyl-L-arginine (L-NMMA; 3 x 10^{-4}M, an inhibitor of synthesis of NO) or (b) 3-morpholinosydnomine (SIN-1; 10^{-7}M or 10^{-5}M, a donor of NO) or (c) no drugs (controls). Catecholamines in the perfusates were quantitated by HPLC with electrochemical detection. In the presence of L-NMMA the effluxes of catecholamines were significantly increased; this increase versus control was approximately 25% for E and NE and 50% for DA. These increases did not occur when Ca^{2+} was omitted from the perfusates. When SIN-1 was present in perfusates the overflow of DA was significantly decreased but overflows of E and NE showed only minor insignificant decreases. It is concluded that a Ca^{2+}-dependent component of spontaneous overflow of catecholamines is inhibited by NO produced in the adrenal medulla. This inhibition appears to be maximal for NE and E, but not for DA.

Supported by U.S. Army Contract DAMD-93-C-3116-P2.

Text will appear in the abstract book exactly as submitted, reduced to approximately 2/3 size.

1965

MOLECULAR EVIDENCE FOR AN H',K'-ATPASE (HKA) IN VASCULAR SMOOTH MUSCLE CELLS (VSMC). X. Zhao, S. Marrelli, and J. Allen. Sect. of Cardiovascular Sci., Baylor Col. Med., Houston, TX 77030.

Recent functional studies demonstrated evidence of HKA in VSMC. Our laboratory used RT-PCR and Northern analysis techniques to identify the presence and type of HKA in canine VSMC. A set of PCR primers was designed to detect unknown HKA isoforms based on the known gastric, colonic, and toad bladder HKA sequences. RT-PCR generated a product at a predicted 310 bp size from canine stomach tissue total RNA as well as from total RNA of whole carotid artery and primary culture of carotid artery VSMC. Nucleotide and deduced amino acid sequence analyses showed a high percentage of homology to the known HKA sequences. Northern blots were performed using the PCR product and Na',K'-ATPase (NKA) α1 cDNA as probes. Stomach and VSMC samples revealed a strong signal with the HKA probe and a weak signal with the NKA probe. Canine kidney had similar mRNA signals with both probes.

Our studies provide the first molecular evidence and partial nucleotide sequence of HKA. HKA may play important roles in VSMC in maintaining both intracellular pH and K content, and may be important in regulation of vessel tone. (Supported by NIH grant, HL24585.)

1966

EFFECT OF $\alpha\alpha$ CROSS-LINKED HEMOGLOBIN (XL-Hb) ON NOREPINEPHRINE (NE) RELEASE AND CONTRACTION IN FEMORAL ARTERY (FA). L.W.Hunter, G.M. Tyce and D.K. Rorie, Mayo Clinic and Mayo Foundation, Rochester, MN 55905

The aim of this study was to determine if vasoconstriction caused by the hemoglobin-based oxygen carrier XL-Hb is associated with increased release of NE. Helical strips of canine FA were superfused with Krebs-Ringer solution (KR) containing corticosterone, desmethylimiprimine and indomethacin (2 ml/min, 37° C, 20% O_2). After 60-min, superfusate was collected during three 25-min periods, each 60 min apart. In each period transmural stimulation (TS; at 2 Hz for periods 1 and 2; 10 Hz for period 3) was applied between min 5-10. In control vessels KR was applied throughout; in other vessels KR containing XL-Hb (10^{-5} M) or N^{ω}-methyl L-arginine (L-NMMA; $3X10^{-4}$ M) or XL-Hb + L-NMMA was applied 20 min before the second period and was maintained. The NE which overflowed was quantified by HPLC-ED. NE overflow and contractions were expressed as percentage of those measured in the first TS in each vessel. XL-Hb, L-NMMA or XL-Hb together with L-NMMA contracted resting vessels (65%, 64% and 76%, respectively), however concurrent NE overflow was unaffected. Overflow of NE and contractile tensions induced by TS were unchanged by any treatment as compared to controls. Thus in the FA XL-Hb: 1) contracts unstimulated vessels by a mechanism not associated with increased NE release, and 2) is without effect on TS-induced contractions or NE release. Supported by U. S. Army contract DAMD17-93-C-3116-PZ.

1967

THE VASCULAR EFFECTS OF PINACIDIL IN TROUT (*Oncorhynchus mykiss*). Michael P. Smith and Kenneth R. Olson, Department of Biological Sciences, University of Notre Dame and South Bend Center for Medical Education, Notre Dame, IN 46556.

Pinacidil (PNC) is an antihypertensive agent which exerts direct vasorelaxant effects by opening ATP-sensitive potassium channels (K^+_{ATP}) thereby hyperpolarizing vascular smooth muscle. However, K^+ conductance-independent mechanisms of vasorelaxation have also been implicated. We herein present the effects of PNC in *O. mykiss*, to our knowledge the first investigation of K^+_{ATP} pharmacology in fish. *In vitro*, 10^{-6} to 10^{-4}M PNC relaxed rings of the 3rd and 4th efferent branchial and coeliaco-mesenteric arteries as well as the ventral aorta which had been precontracted with arginine vasotocin. This relaxation was variably affected by glyburide (GLY, 10^{-5}M) but not by indomethacin or methylene blue. Interestingly, 10^{-4}M PNC also relaxed otherwise unstimulated rings and rings precontracted with 80mM K^+. *In vivo*, administration of 0.1 to 5.0 mg/kg i.a. PNC immediately but transiently reduced dorsal aortic pressure in conscious cannulated trout by as much as 30% (-10+2 mmHg; n=9), an effect partially inhibited by 5.0 mg/kg i.a. GLY. These studies demonstrate that PNC is indeed vasorelaxant *in vitro* and hypotensive *in vivo* in trout. However, the particular mechanisms of PNC's action require further investigation. (Supported by IBN 9105247).

1968

Carbon Monoxide Mediates the Coronary Vasodilator Effect of Heme in Isolated Rat Hearts. Matthew J. Scholer, Robert A. Johnson and Alberto Nasiletti. Department of Pharmacology, New York Medical College, Valhalla, NY 10595.

Cardiac tissues express heme oxygenase (HO), an enzyme which metabolizes heme to carbon monoxide (CO) and biliverdin. CO is an activator of soluble guanylate cyclase and has been shown to relax vascular smooth muscle. These experiments were designed to test the hypothesis that HO product(s) contribute to the regulation of coronary vascular tone. The hearts of Sprague-Dawley rats were isolated and the coronary vasculature was perfused according to the Langendorff technique at a constant flow rate (8-9 ml/min) with oxygenated Krebs' buffer containing N^{ω}-nitro-L-arginine methyl ester (L-NAME, 50 µM), an inhibitor of nitric oxide synthase, to establish a vasoconstrictor tone. Baseline perfusion pressure after L-NAME administration averaged 133±3 mmHg. Addition of heme-L-lysinate (HLL, 1 µM), a substrate for HO, to the perfusion buffer resulted in a 22±5 mmHg decrease in perfusion pressure after 14 minutes ($p<0.05$). In contrast, this response was not seen with addition of a lysine/solvent vehicle. More importantly, 1 µM HLL did not decrease perfusion pressure during infusion of the HO inhibitor zinc deuteroporphyrin 2,4-bis glycol (ZnDPBG) at a concentration of 50 µM. Additionally, bolus injection of Krebs' solution (1ml) saturated with carbon monoxide gas caused a 21±2 mmHg decrease in perfusion pressure. This study shows that heme elicits coronary vasodilation via a heme oxygenase-dependent mechanism. We suggest that carbon monoxide is the HO product responsible for the vasodilatory response to heme in the isolated rat heart.

GENE EXPRESSION/GENE THERAPY II (1969-1970)

1969

IDENTIFICATION OF REGULATED GENES IN RAT HEART AFTER MYOCARDIAL INFARCTION Y.-Z. Zhu, Y.-C. Zhu, M. Stoll, Th. Unger (SPON: M. Morris), Dept. of Pharmacol., Univ. of Kiel, Germany.

In order to investigate the gene expression after myocardial infarction (MI), we have used a RNA fingerprinting method, originally developed by Liang and Pardee (Science 1992; 257: 967-971), a so-called differential display reverse transcription polymerase chain reaction (DDRT-PCR). MI was induced by ligation of the left anterior descending coronary artery (LAD) in the rat. Total RNA was extracted from the right ventricle (RV) 6 weeks after MI. Fifty differentially regulated cDNA fragments were obtained after amplification with the arbitrary down-stream primer T11CT. Eight candidate cDNA fragments were extracted and chosen for further analysis. The reamplified PCR-fragments were subcloned and sequenced. The differential expression of the clones of interest was confirmed on Northern blots. Sequence analysis demonstrated that two of these clones corresponded to unknown genes, whereas the other four represented known genes not previously associated with MI. The latter group includes among others, the mouse interleukin-4 receptor gene, rat ferritin mRNA and T-cell receptor beta chain V.

Our results suggest that myocardial gene expression is strongly altered in the phase of remodeling after MI. The genes isolated represent genes which have not yet been connected with MI and may be instrumental in the remodeling phase.

1970

DECREASED COX I mRNA IS TRANSCRIPTIONALLY REGULATED IN THE SENESCENT RABBIT MYOCARDIUM. H. Matsuda, J.D. McCully, L.E. Dunphy, S. Levitsky. Div. of Cardiothoracic Surgery, Deaconess Hospital & Harvard Medical School, Boston, MA 02215

Cytochrome oxidase I (COX I) mRNA levels are decreased in the aged as compared to the mature rabbit heart. To discriminate if this difference was the result of either decreased synthesis or increased degradation of mitochondrial RNA, purified mitochondria from mature (15-20 weeks, n=7) and aged (>130 weeks, n=7) rabbits hearts were isolated. Mitochondrial transcription rates evaluated by [^{32}P]UTP incorporation indicated lower ($p<0.05$) incorporation in aged as compared to the mature heart (19.2±1 vs. 28.1±2.2 x10^4c.p.m. at 60 minutes incubation). COX I mRNA transcription measured by run-on transcription and Northern hybridization was decreased ($p<0.05$, n=5) in the aged as compared to the mature heart. Mitochondrial COX I mRNA is decreased due to decreased mitochondrial transcription in the aged heart.

Supported by grants from NIH (HL29077) and AHA (95006300).

TITLE: ROLE OF NITRIC OXIDE IN MODULATION OF EVOKED CATECHOLAMINE EFFLUX FROM CANINE ADRENAL GLAND

AUTHS: RD Barnes, MD; LE Ward, BS; GM Tyce, PhD; DK Rorie, MD PhD

AFFIL: Depts. of Anesthesiology, Physiology and Biophysics, Mayo Clinic/Foundation, Rochester, MN

INTRODUCTION: Whether nitric oxide modulates efflux of catecholamines from the adrenal gland during nicotinic stimulation was studied. Evoked effluxes were compared in the presence and absence of a nitric oxide synthase (NOS) inhibitor N^G-monomethyl-L-arginine (L-NMMA, $3x10^{-4}$M), an analogue of L-arginine.

METHODS: Isolated canine adrenal glands were perfused retrogradely with Krebs-Ringer solution[1]. After 60-min stabilization, perfusate was collected during (a) a 10-min basal period, (b) a 2-min stimulation with a "low" ($3x10^{-6}$M) or a "high" ($5x10^{-5}$M) dose of 1,1-dimethyl-4-phenylpiperazinium (DMPP), a nicotinic agonist, (c) an 8-min post-stimulation, and (d) a 30-min stabilization period. This stimulation sequence was repeated three times (S_1, S_2, S_3). In some studies L-NMMA was added to the perfusate before the third sequence of collections. Epinephrine (E), norepinephrine (NE) and dopamine (DA) in perfusates were quantified by HPLC. Net evoked catecholamine efflux was calculated by subtracting prior basal efflux from total evoked efflux. The ratios of net evoked catecholamine effluxes in S_3 expressed as a percentage of effluxes in S_2 were compared in the presence vs. the absence of L-NMMA.

RESULTS: Effluxes (as ng/min) of E, NE, and DA during S_2 were: 2050±191, 264±50 and 23±3 with low dose of DMPP, and 4306±664, 1215±197 and 43±3 with high dose of DMPP respectively.

S_3/S_2 Ratios in Presence and Absence of L-NMMA

	Low Dose DMPP		High Dose DMPP	
	Control	with L-NMMA	Control	with L-NMMA
E	80.5±18.8%	112.4±13.3%	73.8±3.1%	58.0±5.7%*
NE	56.0±7.2%	125.7±13.3%*	61.5±2.3%	58.9±5.6%
DA	73.7±5.8%	121.1±19.5%*	82.7±4.6%	63.3±4.5%*

*Significantly different from control.

During low dose nicotinic stimulation L-NMMA increased evoked effluxes of NE and DA but not of E. During high dose nicotinic stimulation L-NMMA had opposite, but smaller effects: effluxes of E and DA were decreased whereas NE efflux was unchanged.

CONCLUSION: In the perfused canine adrenal gland, inhibition of nitric oxide production affected evoked catecholamine efflux differently depending on the degree of nicotinic stimulation. Thus, evidence suggested that nitric oxide diminished evoked efflux of catecholamines at low levels of nicotinic stimulation but increased efflux at higher levels. (At both low and high intensity stimulation the effects of L-NMMA were different for E, NE and DA.) These data implicate a role for nitric oxide in modulation of catecholamine release in response to stressors e.g. hemorrhage or hypoxia. Understanding all actions of nitric oxide, nitric oxide donors and NOS inhibitors is also important because of their current and potential clinical applications.

REFERENCES: [1]Am J Physiol 260:R589-R599, 1991.

THE ADRENAL GLAND AS A SOURCE OF DOPA AND OF CATECHOLAMINE METABOLITES

Tyce GM[1], Chritton SL[1], Barnes RD[2], Ward LE[2], Hunter LW[2], Rorie DK[2]
Depts of [1]Physiology and Biophysics and [2]Anesthesiology, Mayo Clinic/Foundation
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Background DOPA, catecholamines and their metabolites in plasma have been proposed as indices of activity in peripheral sympathetic nerves. However, the adrenal gland is also a rich source of catecholamines. The purpose of this study was to examine the characteristics of release and reuptake of these compounds in dog adrenal gland. The effects of cocaine, an inhibitor of the neuronal reuptake of norepinephrine (NE) and of nitric oxide (NO), a modulator of NE release, on catecholamine releases were studied.

Methods Isolated dog adrenal glands were perfused *ex situ* with oxygenated Krebs-Ringer solution at 37°. Perfusates were collected before, during and after a 2-min stimulation with carbachol ($3x10^{-5}$M) or 1,1-dimethyl-4-phenylpiperazinium (DMPP, $3x10^{-5}$M). In some experiments cocaine (10^{-5}M) or an inhibitor of NO synthesis (N^G-monomethyl-L-arginine [L-NMMA], $3x10^{-4}$M) was added. Epinephrine (E), NE, dopamine (DA), DOPA, metanephrine (MN), noremetanephrine (NMN), 3-methoxytyramine (3MT), 3,4-dihydroxyphenylglycol (DHPG), 3,4-dihydroxyphenylacetic acid (DOPAC) and 3-methoxy, 4-hydroxyphenylglycol (MHPG) were quantified in perfusates by HPLC with electrochemical detection.

Results and Interpretation During the first 60 min of perfusion the concentrations of E, NE, DA and DOPA declined exponentially; the levels of the metabolites did not change. After 60 min, the mean (±SEM) overflows of E, NE, DA, DOPA, MN, NMN, DOPEG , DOPAC and MHPG were 4500±2000, 680±200, 57±10, 18±1, 230±80, 93±60, 380±100, 36±10 and 19±4 pmoles/min respectively. Carbachol increased the releases of E, NE, DA and DOPA, but not of the metabolites. Cocaine had no effects on basal or evoked releases of any of the compounds. L-NMMA increased basal, but decreased evoked, releases of E, NE, and DA.

Conclusions In the dog adrenal gland (1) DOPA was released in a similar manner to the catecholamines; (2) Reuptake of catecholamines could not be demonstrated; (3) Because DOPEG was unaffected by the presence of cocaine, it was probably produced subsequent to translocation of NE and E from chromaffin granules into cytoplasm; (4) *O*-Methylated metabolites of catecholamines were abundant; (5) NO modulated catecholamine release and the effects were opposite under basal and evoked conditions.

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12 July 1996

Dr. Duane K. Rorie
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Re : BIOGENIC AMINES TN-383

Dear Dr. Duane K. Rorie :

Thank you for submitting your paper on "Transmural stimulation of mesenteric artery in the presence of cross-linked hemoglobin produces a compound with chromatographic characteristics similar to dopamine" by Larry W. Hunter, Gertrude M. Tyce, Linda M. Benson, Stephen Maylor and Duane K. Rorie.
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Transmural stimulation of mesenteric artery in the presence of cross-linked hemoglobin produces a compound with chromatographic characteristics similar to dopamine

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Key Words: Dopamine; hemoglobin; Blood substitutes; vasoconstriction; blood vessels

Running Title: A Dopamine-like compound from hemoglobin

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ABSTRACT

When sympathetic nerve endings in isolated canine mesenteric arteries were depolarized electrically, a compound appearing to be dopamine, was released frequency-dependently. Production of the putative dopamine occurred only in arteries exposed to αα cross-linked hemoglobin. The aim of this study was to determine whether this compound was authentic dopamine. Several chromatographic characteristics of the unknown compound were identical to those of dopamine; adsorption onto Sep-Pak C_{18} cartridges, and isographic elution on a reversed-phase HPLC column. However, further analysis revealed that the compound did not adsorb onto neutral alumina or onto cationic-exchange resin as did dopamine, and that its voltammetric properties were not identical to those of dopamine. Subsequently, the compound was found to be produced in Krebs-Ringer solution in the absence of artery, provided αα cross-linked hemoglobin, oxygen and an electric current were supplied. Similar results were obtained when other proteins were substituted for αα cross-linked hemoglobin. It is concluded that the compound released from mesenteric artery by αα cross-linked hemoglobin was not dopamine.

INTRODUCTION

During initial studies, a compound, tentatively identified as dopamine (DA), overflowed from superfused blood vessels subjected to transmural electrical stimulation. However the compound was found only in vessels which were exposed to αα cross-linked hemoglobin (XL-Hb), a proposed blood substitute for use in transfusion therapy. XL-Hb, like free native hemoglobin (Hb), has a pressor effect when transfused (Schultz *et al.*, 1993; Winslow, 1992), a property which decreases its utility as a blood substitute. However, among different vascular beds, there is heterogeneity in contractile responses to XL-Hb (Sharma *et al.*, 1994). The pressor effect of XL-Hb appears to result primarily from constriction of resistance vessels, although the mechanisms involved are not understood fully. The capacity of XL-Hb to scavenge the vasodilator nitric oxide (NO) (Martin *et al.*, 1985; Schultz *et al.*, 1993), is probably one causal factor. Also, XL-Hb may induce the release of endothelin (Schultz *et al.*, 1993) or vasoconstrictor prostaglandins (Toda, 1990). Release of the vasodilator DA from vascular nerve endings would potentially be beneficial in counteracting the vasoconstriction induced by XL-Hb. The hypothesis proposed was that the heterogeneous responses in blood flow among different vascular beds upon administration of XL-Hb might be due, in part, to the differential release of DA. Therefore the aim of this study was to characterize the compound released to determine whether it was endogenous DA.

MATERIALS AND METHODS

Materials

XL-Hb used was prepared by the U.S. Army Medical Research and Development Command from stroma-free Hb obtained from outdated human blood. Preparation included modification with bis (3,5-dibromosalicyl) fumarate (Snyder *et al.*, 1987). Neutral alumina was purchased from ICN (Costa Mesa, CA), and Bio-Rex 70 was from Bio-Rad Laboratories (Hercules, CA., USA). Catecholamine standards and proteins were purchased from Sigma (St. Louis, MO., USA). Sep-Pak C_{18} cartridges were from Waters Assoc. (Milford, MA., USA).

Artery superfusion

Tissue preparation. These studies were approved by the Institutional Animal Care and Use Committee. Mesenteric arteries were obtained from anesthetized adult male or female mongrel dogs. Other tissues were obtained simultaneously from each dog for study in seven other Mayo research laboratories. Helical strips of the arteries were prepared and mounted for superfusion as described previously (Hunter *et al.*, 1992, 1996). The strips were superfused at 2 ml/min with Krebs-Ringer (K-R) solution (Muldoon, *et al.*, 1979) which was aerated with 95% O_2, 5% CO_2 and maintained at 37° C. After mounting, the strips were equilibrated for 60 min. Subsequently, superfusate was collected in two 5-min intervals, with transmural stimulation (TMS) at 2 Hz (10 V, 0.2 mSec) being applied only during the second interval. The strips were then equilibrated for another 60-min, then superfusate was collected for

three more 5-min intervals, with 12 Hz TMS being applied during the second of these intervals.

Chromatography. The superfusate which passed over each strip of artery during each 5-min interval was pulled by pump through a Sep-Pak C_{18} cartridge attached to the bottom of the superfusion chamber. The cartridge was then rinsed and the unknown compound was eluted with 2 ml HPLC mobile-phase buffer. The sample was then injected onto a reverse-phase HPLC system which utilized a coulometric detector (Hunter *et at.*, 1992). Endogenous NE, as well as its major metabolite dihydroxyphenylglycol (DHPG), also overflowed into the superfusate and were eluted from the Sep-Paks together with the unknown compound. Neither NE nor DHPG was quantified.

In some experiments, the superfusate which passed over the strips of vessel during the 12 Hz stimulation interval was collected into a beaker, then divided equally. One-half was processed by Sep-Pak as described above to confirm the presence of the unknown compound. The other half was processed through a column of neutral alumina, then through a column of Bio-Rex-70 cation exchange resin to determine whether the compound had the chromatographic characteristics of a catecholamine (Valori *et al*, 1969). Some of these K-R samples were passed through only one of the columns to determine if the compound contained either a catechol or an amine group, respectively. Alumina and Bio-Rex 70 column eluates were injected onto the same HPLC system used for the Sep-Pak eluates.

Non-artery experiments

In a second series of experiments, the unknown compound was generated in the absence of tissue, using conventional tissue chambers designed for measuring isometric contractions in blood vessel ring preparations. Twelve ml of K-R was added to each chamber and aerated continuously. After 20 min equilibration, an electrical current (10 V, 12 Hz, 0.2 mSec) was passed through the K-R by means of two small platinum plates designed to be placed on either side of rings of tissues. After 10 min of stimulation, 10 ml of K-R was removed from each chamber and the unknown compound was assayed by Sep-Pak as described above. Experiments were done using K-R alone as well as with K-R containing XL-Hb, bovine Hb, cyanomet Hb, myoglobin, apomyoglobin, bovine serum albumin (BSA), chicken egg albumin or trypsin inhibitor from soybean; each at a concentration of 2×10^{-5} M. In some experiments using XL-Hb, the O_2 in the aerating gas was replaced with N_2.

Voltammetry

In other experiments, the voltammetric behavior of the unknown compound, produced from XL-Hb by each of the two methods described above, was compared to that of authentic DA. Hydrodynamic voltammograms were generated by HPLC, using Sep-Pak eluates of: (1) 10 ml K-R spiked with 2 ng authentic DA; (2) the unknown compound produced by superfusion and stimulation of a mesenteric artery at 12 Hz for 5 min in the presence of XL-Hb (2×10^{-5} M); and (3) the unknown compound produced in a tissue chamber containing XL-Hb (2

X 10^{-5} M) which had been stimulated at 12 Hz for 10 min in the absence of artery. For each sample, the HPLC detector responses between -50 to 200 mV, in 25 mV increments, were measured and expressed as the percent of the maximum response.

Mass spectrometry

In another set of tissue chamber experiments, identical to those described above, the presence of the unknown compound in samples generated by stimulation of K-R containing XL-Hb was first ascertained by HPLC. The balance of each sample was then analyzed by mass spectrometry (MS) to establish the presence of DA. Several ionization methods were utilized, including electron impact (EI), chemical ionization (CI), electrospray ionization (ESI), and atmospheric chemical ionization (APCI) (Suelter and Watson, 1990). Analyses were performed on double-focusing sector instruments (Finnigan-MAT 900 or Finnigan-MAT 95Q; Finnigan, Bremen, Germany) using authentic DA standards to optimize operating parameters for sensitivity.

RESULTS

Small amounts of NE and its major metabolite DHPG overflowed from mesenteric artery strips during basal conditions (Fig. 1B), and the overflows were increased frequency-dependently when the vascular nerve endings were depolarized by TMS (Figs. 1C, 1D). During the stimulations, and immediately after the 12 Hz stimulation, large amounts of a compound isographic with DA also overflowed from the strips exposed to XL-Hb (Figs. 1C-1E), but not from control strips (Fig. 1F).

Several superfusate samples, collected from artery strips exposed to XL-Hb during the 12 Hz interval, were assayed for catecholamines using alumina, Biorex-70 columns; with the columns being used separately as well as in tandem. No peak, isographic with DA, was found in these samples, even though a substantial peak was always found in duplicate samples isolated on Sep-Paks (Fig. 1D).

In experiments done in tissue baths in the absence of artery, the unknown compound was not produced when K-R was aerated with 95% O_2, 5% CO_2, and stimulated at 12 Hz for 10 min (Fig. 2A). However it was produced when XL-Hb (2×10^{-5} M) was added to the K-R and the same stimulation applied (Fig. 2B). It was also produced in samples in which the XL-Hb was replaced with other proteins of the same concentration: bovine Hb, cyanomet Hb, myoglobin, apomyoglobin, BSA (Figs. 2C-2G). When chicken egg albumin or trypsin inhibitor from soybean were used the peaks were also in evidence (data not shown). The unknown compound was not found in

stimulated XL-Hb samples in which the O_2 was replaced with N_2 (Fig. 2H).

Between -50 and 200 mV, the voltammetric behavior of the unknown compound generated in the absence of artery was identical to that of the compound generated when artery was present, but showed some differences from that of authentic DA (Figure 3).

Analysis of Sep-Pak eluates from samples which contained the unknown compound, as verified by HPLC, yielded high counts of background ions which interfered with MS detection. In negative mode APCI-MS, the limits of sensitivity for DA were 0.1-0.5 ng per sample. APCI-MS in combination with direct on-line reverse-phase HPLC separation prior to MS detection (HPLC-APCI-MS) provided the greatest selectivity for DA. However, DA was not found in samples which had been stimulated, or in unstimulated controls. The results of these and many other similar experiments indicated that the unknown compound did not have DA-like characteristics during MS analysis.

DISCUSSION

Results of preliminary studies were consistent with the concept that XL-Hb induces or augments the release of endogenous DA from vascular nerve endings. This was a compelling avenue to pursue since, if substantiated, it might help explain the differences among blood vessels in their contractile response to XL-Hb (Sharma *et al*, 1994). In fact, several findings of the present study support this hypothesis. The compound tentatively identified as DA was isographic with authentic DA on a reversed-phase HPLC system commonly used for catecholamines. Also, it was adsorbed onto and eluted from Sep-Pak C_{18} cartridges under the same conditions as was DA. The height of the chromatographic peak which co-eluted with DA on HPLC was increased frequency-dependently by TMS. Endogenous DA is, in fact, released in a frequency-dependent manner, although in small amounts, from isolated segments of portal vein upon TMS (Hunter *et al.*, 1992). Also DA is released from isolated canine mesenteric artery during prolonged nerve depolarization (Soares-da-Silva, 1987).

However, subsequent analysis revealed characteristics of the unknown compound which were significantly divergent from those of authentic DA. For example, the voltammetric behavior of the unknown compound was not identical that of DA. Further, the compound was not adsorbed onto neutral alumina or onto weak cation-exchange resin, indicating that it was neither a catechol nor an amine as is DA (Valori, *et al.*, 1969). Moreover, the unknown compound was not derived directly from mesenteric artery since it

was produced in its absence. The compound which was generated during the superfusion of artery strips in the presence of XL-Hb was likely the same compound which was generated in the absence of artery in the tissue chambers containing only K-R and XL-Hb, since their chromatographic and voltammetric behaviors were identical.

A number of mass spectrometric ionization techniques were utilized in both the positive and negative ion mode in an attempt to obtain molecular weight and structural information on the compound. In all cases, it was not possible to unequivocally identify a single component by mass spectrometry after collection of the appropriate fraction from HPLC. This could be due to several factors including: (1) amounts of the compound produced were below the limits of detection by MS, (2) the compound does not posess functional groups that would result in any significant ionization or, (3) the compound is a small molecular weight component. Such compounds are sometimes difficult to detect due to the substantive amount of chemical noise below 200-300 Da.

The identity of the unknown compound was not ascertained, although several findings do give an insight into some of its characteristics. It was not derived specifically from XL-Hb since it was produced from native bovine Hb and from cyanomet Hb as well as from myoglobin. Although these compounds are all heme proteins, the unknown compound was not derived from heme since it was produced from apomyoglobin, which lacks the heme moiety. It was also produced in the presence of other non-heme proteins, including BSA, and notably, chicken egg albumin and trypsin inhibitor from

soybean, two proteins not derived from blood.

In several experiments, we examined the possibility that the unknown compound might influence the release or disposition of NE or might affect contraction in isolated blood vessels. However, no effects on the release or disposition of NE could be demonstrated, thus these areas of investigation were not pursued, particularly since no agonist activity on blood vessels could be demonstrated.

Although the unknown compound detected in these experiments was not DA, it may have been a derivitive of DA or of the DA precursor 3,4-dihydroxyphenylalanine (DOPA). DOPA has widespread occurrence in animals and in plants (Banwart *et al.*, 1989), and it can be converted nonenzymatically to DA (Vogel, 1969). DA and DOPA autoxidize to aminochromes, quinones and other derivitives (Graham, 1978). Catecholamines and their derivitives bind to proteins (Banwart *et al.*, 1989; Boomsma *et al.*, 1991). Therefore, it is possible that the unknown compound originated from an adduct of a DA derivitive with the various proteins studied, and was cleaved and released as an oxidation product during stimulation.

In summary, a compound which appeared to be DA was released from depolarized nerve endings in isolated canine mesenteric artery strips which were exposed to XL-Hb. These studies show that the compound was not DA; its identity was not determined.

Acknowledgements

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FIGURE LEGENDS

FIG. 1. HPLC chromatograms illustrating the overflow of putative DA in mesenteric artery strips superfused *in vitro*. Details are given in Materials and Methods.

Fig. 2. HPLC chromatograms scanned between 10 and 18 min, illustrating the production of an unknown compound which co-eluted with DA (see Fig. 1) in the presence of various proteins.

FIG. 3. Hydrodynamic voltammogram of authentic DA (0.1 ng); comparison with that of an unknown compound derived from K-R superfusate of mesenteric artery which was stimulated at 12 Hz for 5 min in the presence of XL-Hb (2×10^{-5} M), as well as from K-R with the same concentration of XL-Hb, stimulated in the absence of artery.

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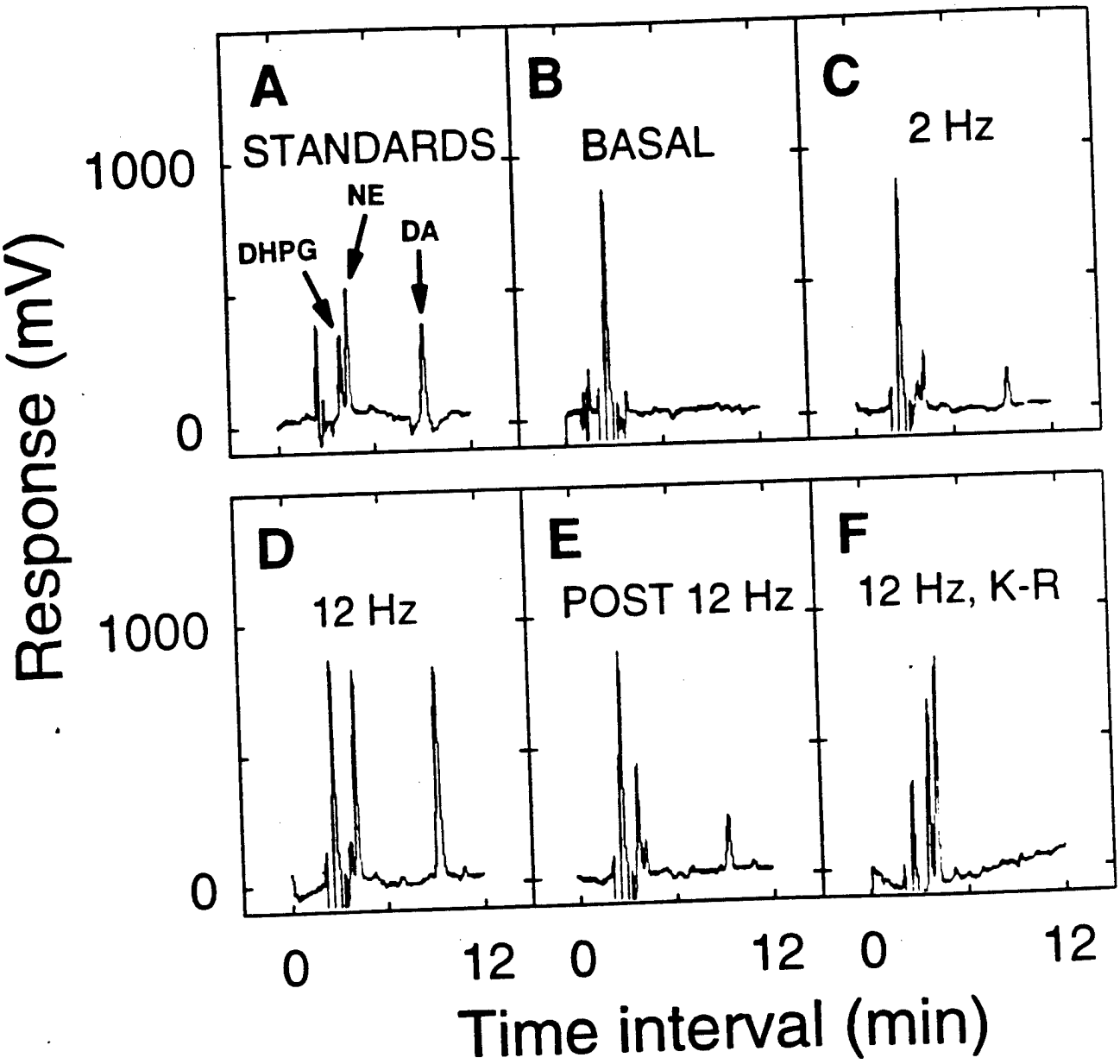
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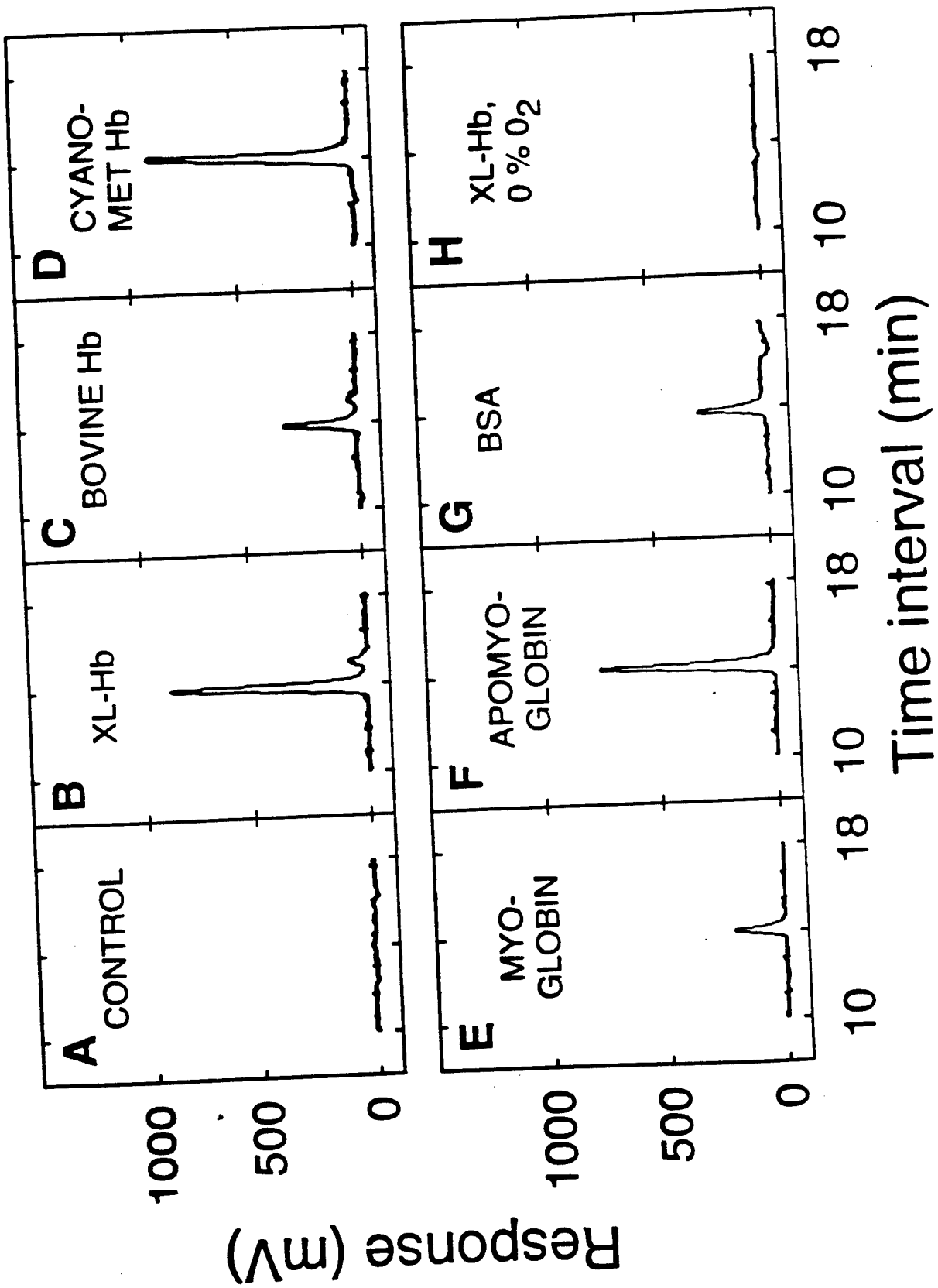
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D CYANO-MET Hb
E MYO-GLOBIN
F APOMYO-GLOBIN
G BSA
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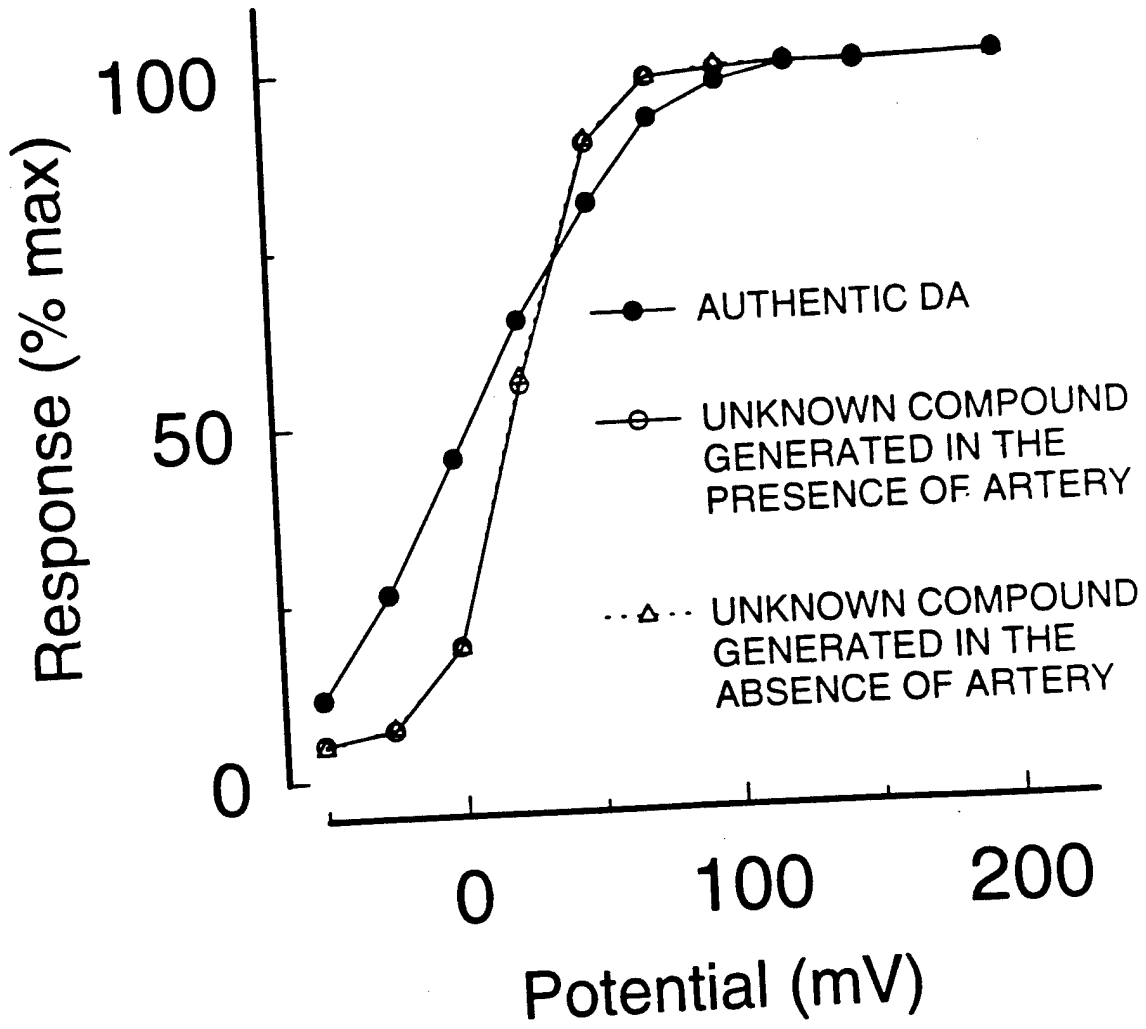
Figure 3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10 July 1996

Dear Professor Rorie,

Re: **MS PA 14-96, Ward et al.**

I am pleased to say that your manuscript entitled *"Nitric oxide reduces basal efflux of catechoclamines from perfused dog adrenal glands"* has been seen by a reviewer and is now considered to be acceptable for publication. The reviewer did however comment that the number of dogs or adrenal glands used in the study has still not been included in the revised manuscript. I would be very grateful if you would let me have this information so that I can insert it in the manuscript prior to sending it for publication. The reviewer again raised the issue of publication of HPLC traces but in my opinion their inclusion in the manuscript is not necessary.

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NITRIC OXIDE REDUCES BASAL EFFLUX OF CATECHOLAMINES FROM PERFUSED DOG ADRENAL GLANDS

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Key Words: Nitric oxide, catecholamine efflux, adrenal gland, met-enkephalin, preproenkephalin, peptides

Running Title: Nitric oxide reduces adrenal catechol efflux

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ABSTRACT

Norepinephrine, epinephrine, dopamine, and both the free and extended forms of [met]enkephalin spontaneously efflux from adrenal glands under basal conditions. The present study was done to determine whether nitric oxide has a regulatory role in these effluxes. Isolated adrenal glands from mongrel dogs were perfused retrogradely with Krebs-Ringer solution. In some experiments N^G-monomethyl-L-arginine ($3x10^{-4}M$), an inhibitor of nitric oxide synthesis, was added to the perfusate. In other experiments one of the nitric oxide donors, 3-morpholinosydnonimine ($10^{-7}M$ or $10^{-5}M$) or sodium nitroprusside ($10^{-6}M$ or $10^{-4}M$) was added. Norepinephrine, epinephrine, dopamine and their metabolites 3,4-dihydroxyphenylglycol and 3,4-dihydroxyphenylacetic acid in perfusates were quantitated by high performance liquid chromatography with electrochemical detection and in some experiments the [met]enkephalins were determined by radioimmunoassay. In the presence of N^G-monomethyl-L-arginine, the basal effluxes of norepinephrine, epinephrine, and dopamine were significantly increased from control, but the effluxes of the free and extended forms of the [met]enkephalins were not changed. The effects of N^G-monomethyl-L-arginine on catecholamine efflux were reversed in the presence of L-arginine ($10^{-3}M$). Sodium nitroprusside ($10^{-6}M$) inhibited effluxes of norepinephrine and epinephrine and 3-morpholinosydnonimine had no effect on these effluxes. Dopamine efflux appeared to be under different controls from those of norepinephrine and epinephrine since dopamine efflux was unaffected by sodium nitroprusside and was decreased over time by 3-morpholinosydnonimine ($10^{-7}M$). It is concluded that endogenously produced nitric oxide inhibits the basal efflux of norepinephrine, epinephrine, and dopamine from isolated dog adrenal glands; this inhibition appears to be near maximal for norepinephrine and epinephrine but not for dopamine.

INTRODUCTION

Epinephrine (EPI), norepinephrine (NE), dopamine (DA) and the neuropeptide [met]enkephalin (ME) spontaneously efflux from the adrenal gland in several species [8,15,20]. Nitric oxide (NO), an important regulatory molecule, has previously been ascribed a role in the modulation of catecholamine release at peripheral sympathetic nerve terminals [11,26,39,41]. Controversy exists concerning the presence of the enzyme, nitric oxide synthase (NOS), within the chromaffin cells [28,31], but the enzyme has been detected in ganglion cells present in the adrenal medulla [38]. Further, the adrenal medulla is a highly vascularized tissue, thus NOS would be expected to be present in the endothelial cells lining the vascular space in the medulla although its presence in these cells has not been demonstrated. NO produced by these endothelial cells or by ganglion cells could help modulate release of catecholamines. An aim of this study was to determine whether NO affects the efflux of catecholamines as well as of ME from the adrenal medulla during basal conditions.

The studies were done using isolated perfused adrenal glands from dogs. The effluxes of EPI, NE and DA were measured using high performance liquid chromatography (HPLC) with electrochemical detection. Substantial amounts of 3,4-dihydroxyphenylglycol (DOPEG) and 3,4-dihydroxyphenylacetic acid (DOPAC), metabolites of the catecholamines, have also been shown to efflux from perfused dog adrenal gland [8] and the amounts of these compounds in perfusates were also measured in this study. Effluxes of catecholamines were measured in the presence or absence of Ca^{2+}, of N^G-monomethyl-L-arginine (L-NMMA), an inhibitor of NOS devoid of muscarinic activity [5], of L-NMMA in the presence of L-arginine, and of the NO donors sodium nitroprusside (SNP) or 3-morpholinosydnonimine (SIN-1).

The pentapeptide ME is co-stored with catecholamines in granules in chromaffin cells as well as in terminals of the splanchnic nerve which innervates the gland [7,42]. During electrical stimulation of the splanchnic nerve and during perfusions with solutions containing acetylcholine or high potassium chloride, ME is released concurrently with catecholamines from the cat adrenal gland [7]. ME is derived from preproenkephalin, a large precursor prohormone, by enzymatic

processing. Trypsinized ME (TME), an extended storage form of ME, is released from cat adrenal gland together with free ME [16] and the proportion of TME to ME varies in different physiological and pharmacological conditions. It has been proposed that TME may itself be active at opioid receptors [16]. In the present study, experiments were done to ascertain whether TME effluxed from dog adrenal gland and, if such an efflux could be demonstrated, whether the efflux was affected by NO.

METHODS AND MATERIALS

Perfusions of adrenal glands.

The protocol used in these studies was approved by the Institutional Animal Care and Use Committee. Mongrel dogs of either sex and unmatched for age were anesthetized with 30 mg/kg sodium pentobarbital, i.v., the adrenal glands removed and placed in ice-cold Krebs-Ringer solution (K-R) [10,35].

One adrenal gland per dog was perfused retrogradely with K-R via a cannula secured within the adrenolumbar vein [33]. The left adrenal gland was used because of the ease of cannulation. To allow the perfusate to exit, a 3 mm slit was made through the cortex to the medulla at the end of each lobe. The K-R, aerated continuously with 95% O_2 and 5% CO_2 and maintained at 37°C, was pumped through the adrenal gland using a constant flow pump set at 1.5 ml/min. Perfusates were collected in graduated cylinders on ice. When assaying for peptides an aliquot of perfusate was removed and frozen; 5% sodium metabisulfite (10 ul/ml of perfusate) and 2N HCL (33 ul/ml of perfusate) were added to the remainder of the perfusate. When spontaneous effluxes of the ME and TME were to be measured together with the catecholamines, bovine serum albumin (BSA; 50 mg/100 ml) was added to the K-R in an effort to prevent plating of the neuropeptides on glass surfaces. No difference was found in the data on catecholamine effluxes in the groups of experiments with or without BSA thus the data were combined.

Adrenal glands were perfused for a 160-min stabilization period using K-R, then an initial basal sample of perfusate was collected. Perfusion was continued with (a) K-R (controls), (b) L-NMMA ($3x10^{-4}$M) in K-R applied at the beginning of the second collection period and continued throughout all subsequent collections, or (c) SNP (10^{-6}M or 10^{-4}M) or SIN-1 (10^{-7}M or 10^{-5}M) in K-R. When SNP or SIN-1 was used, each was added only during a single 10-min collection period. In other experiments, adrenal glands were perfused for a 160-min stabilization period with Ca^{2+}-free K-R containing 0.1mM ethyleneglycol-bis(β-aminoethylether)N,N-tetraacetic acid (EGTA) [22]. After an initial basal sample of perfusate was collected perfusion was continued with Ca^{2+}-free K-R either in the absence or presence of L-NMMA (3×10^{-4}M). In further experiments, the effects of L-

NMMA were studied in perfusions in which L-arginine (10^{-3}M) had been added to the K-R 30 min before the L-NMMA.

After the initial stabilization period perfusates were collected in 10-min intervals for a total of 70 min. In most experiments perfusate samples collected between the 30th and 60th min were combined to form a single sample for analysis (i.e. that in interval 4).

Measurement of catecholamines, catecholamine metabolites and peptides in perfusate.

EPI, NE, DA, DOPEG, and DOPAC in perfusates were quantitated by comparison to those of known standards using a HPLC with electrochemical detection. All samples were diluted prior to injection on the HPLC system (e.g. 1 to 125 for determination of DOPEG, NE and EPI and 1 to 5 for determination of DA and DOPAC). Analytes were measured in perfusates after reverse-phase separation on a C-18 analytical column. The mobile phase used for separation of catecholamines on the HPLC contained 70 mM sodium dihydrogen phosphate, 0.4-0.8 mM heptanesulfonic acid (depending on the age of the column), 0.2 mM ethylenedinitrilodisodium tetraacetic acid (EDTA), and 0.15% (wt/vol) trifluoroacetic acid (TFA) at a pH of 2.8. The HPLC system used was similar to that described previously [22], except that the potentials on the 5011 dual analytical cell (ESA, Inc., Bedford, MA, U.S.A.) were set to +150 mV on the first cell and -400 mV on the second cell. Sample detection was measured on the second cell after oxidation on the first.

ME and TME were measured in perfusates by radioimmunoassay as described by Chritton et al. [10] and Lucas and Yaksh [25].

Statistics

The effluxes of catechols, ME and TME in intervals 2-5 were expressed relative to those in the initial sample collected before the introduction of drugs. The data shown are the means ±SEM for the ratios and absolute values of five to nine experiments per group unless stated otherwise. Differences in the effluxes of catechols over time for the various treatments were tested using a one-way analysis of variance with repeated measures followed by a paired t-test of differences in the effluxes in intervals 2-5 versus those in interval 1. Differences in the effluxes of catechols from adrenal glands between control and treated groups were tested for statistical significance using a two-

way analysis of variance with repeated measures. Differences in the efflux of catechols between control and treated groups during the same interval were determined using an unpaired *t*-test. A *p* value less than 0.05 was considered statistically significant.

Materials.

L-NMMA, L-arginine, SNP, NE, EPI, DOPEG, and EGTA were obtained from Sigma; and SIN-1 from Molecular Probes, Inc.; BSA from ICN Biomedicals, Inc.; and DA and DOPAC from Research Biochemicals International.

RESULTS

Effects of inhibition of NO synthesis on effluxes from the adrenal gland.

The effluxes of EPI, NE and DA during the first 10-min collection interval in the presence of Ca^{2+} were 324.3 ± 141.1, 47.8 ± 18.0 and 4.5 ± 1.3 ng/min respectively (n=5 to 7). In control perfusions the basal effluxes of EPI and NE decreased progressively during the 70 min of the experiment to values which were 77.0 ± 8.2% and 76.1 ± 7.9% respectively of the effluxes in the first 10-min interval (Fig. 1 and 2). The basal effluxes of NE and EPI were decreased significantly with time in intervals 3, 4, and 5 compared to interval 1 (Figs. 1 and 2). By contrast, the efflux of DA did not change with time (Fig. 3). The rates of effluxes of EPI, NE and DA in the presence Ca^{2+} were not significantly changed by treatment with L-NMMA ($3x10^{-4}M$) and L-arginine ($10^{-3}M$) together (Figs. 1-3) or with Ca^{2+}-free perfusate (controls' in Figs. 1-3 compared to those in Table 1).

In the presence of L-NMMA and of Ca^{2+} the effluxes of EPI, NE and DA were increased significantly from those in control (Figs.1-3). The effluxes of EPI and of NE were increased significantly from the corresponding control value for intervals 3, 4, and 5 (Figs. 1 and 2). The efflux of DA was significantly increased from the corresponding control value by the presence of L-NMMA in interval 4 (Fig. 3). There was no significant increases in the effluxes of NE, EPI and DA by L-NMMA when Ca^{2+} was absent from the perfusate (Table 1). L-NMMA in the presence of L-arginine had no effect on catecholamine effluxes versus those in the corresponding controls (Figs. 1-3).

The effluxes of DOPEG and DOPAC in the presence of Ca^{2+} were 24.3 ± 6.1 and 2.5 ± 0.7 ng/min respectively (n=7) during the first 10-min collection interval. In control perfusions the effluxes of DOPEG and DOPAC tended to decrease progressively during the 70 min of the experiment to values which were 90.4 ± 5.9% and 88.1 ± 7.0% respectively of the effluxes in the first 10-min interval but these decreases were not statistically significant (data not shown). No differences could be demonstrated in the basal effluxes of DOPEG and DOPAC nor in the changes with time in the presence versus the absence of Ca^{2+} in the perfusate (data not shown). Further, the decreases in the effluxes of DOPEG and DOPAC were not different from controls in the presence

of L-NMMA irrespective of whether Ca^{2+} was present. However when L-NMMA and Ca^{2+} were present the decrease in the basal efflux of DOPAC was significant with time to values in intervals 3, 4, and 5 of 82.9 ± 5.6, 79.6 ± 8.3 and 77.5 ± 6.3 respectively when compared to interval 1.

In the presence of Ca^{2+} the effluxes of ME and TME from adrenal glands in interval 1 were 1168.7 ± 644.9 and 1192.6 ± 522.7 pg/min, respectively (n=4). The effluxes of ME and TME decreased progressively during the 70 min of perfusion to values which were 85.6 ± 24.7% and 49.7 ± 4.5%, respectively of the effluxes in interval 1 (data not shown). The decreases in effluxes of ME and TME were not significantly different in the presence or absence of Ca^{2+} in the perfusate. ME and TME effluxes were not different from controls in the presence of L-NMMA when either Ca^{2+}-replete or Ca^{2+}-free perfusates were used.

Effects of NO donors on effluxes from the adrenal gland.

The presence of SIN-1 (10^{-7} M) during interval 2 resulted in a significant decrease in the efflux of DA in interval 5 (Fig. 4). The efflux of DA was unchanged in the presence of 10^{-5}M SIN-1 (Fig. 4). The introduction of SIN-1 (10^{-7}M or 10^{-5}M) had no significant effects on the effluxes of NE, EPI, DOPEG and DOPAC (data not shown).

Compared to controls, SNP (10^{-6}M) decreased significantly the effluxes of EPI and NE but not of DA during the 10-min collection interval during which it was present in the perfusate, (Table 2). DOPEG efflux was increased significantly within the 10-min collection immediately after 10^{-6}M SNP had been applied (i.e. to 121.7 ± 7.9% in interval 3 compared to 96.7 ± 7.8% in the same interval in control perfusions). Otherwise SNP (10^{-6}M) had no significant effects on the effluxes of DOPEG or of DOPAC. SNP at 10^{-4}M tended to increase the effluxes of EPI, NE and DA but the changes were not significant (Table 2). The efflux of DOPEG was increased significantly during the application of 10^{-4}M SNP and immediately after (i.e. to 118.9 ± 7.2%, 157.8 ± 8.6%, and 147.7 ± 8.9% in intervals 2, 3 and 4 respectively compared to 97.1 ± 5.0%, 96.7 ± 7.8%, and 90.9 ± 4.5% in the control perfusions). SNP (10^{-4}M) significantly increased the efflux of DOPAC in interval 4 (i.e. to 124.7 ± 13.4% compared to 88.1 ± 7.0% in the control perfusions).

DISCUSSION

Major findings of this study.

Inhibition of synthesis of NO by L-NMMA increased the spontaneous efflux of NE, EPI and DA from perfused dog adrenal glands. This action was reversed in the presence of L-arginine. These data imply that spontaneous efflux of the catecholamines is inhibited by endogenously produced NO. NOS inhibitors at high concentrations also inhibit cyclooxygenases and cytochrome P_{450} enzymes [32]. Thus the specificity of L-NMMA in these experiments could be questioned. However, the action of L-NMMA was overcome by addition of L-arginine suggesting strongly that L-NMMA effects were mediated via the NOS pathway. The inhibition appears to be near maximal for NE and EPI because the production of NO using the donor SIN-1 did not further inhibit the effluxes of NE and EPI. On the other hand, the addition of the NO donor SNP at 10^{-6}M did significantly inhibit both NE and EPI effluxes.

The efflux of DA appeared to be controlled differently from the effluxes of NE and EPI in that DA efflux was inhibited by SIN-1 at 10^{-7}M (although this inhibition was late in onset) but was unaffected by SNP. We have previously shown that DA was also released preferentially to E and NE from dog adrenal gland when stimulation was by nicotinic and muscarinic agonists [10]. This result would not be expected if DA were stored in the gland only as a precursor of NE and E. DA is stored in adrenal gland in small granule cells [23] which are relatively few in number. The preferential release of DA could be due to the small size of these DA cells. As pointed out by Chritton et al. [10], if the concentration and activity of the exocytotic mechanism and vesicular storage in a theoretical cell are held constant, then an increase in cell size will lead to a greater proportional increase in the volume of storage than in the surface-limited exocytotic mechanism. Thus, release could be expected to be greater from a small than from a large cell. Although the major fraction of basal efflux from adrenal gland is nonexocytotic, the same principles may apply to nonexocytotic and exocytotic effluxes.

It is currently widely accepted that actions of NO are mediated through an increase in guanylate cyclase activity in target cells [30]. It has been recognized that activation of muscarinic

receptors also results in an increase in cyclic GMP in chromaffin cells [37] so stimulation by NO may mimic muscarinic stimulation. Muscarinic stimulation has variously been shown to directly evoke release of catecholamines from the adrenal medulla but importantly also to down regulate the release evoked by nicotinic agonists [10,36]. Further studies are needed to determine whether the putative actions of NO in inhibiting basal effluxes of catecholamines from dog adrenal glands are mediated by changes in cyclic GMP production.

Our data showing that NO inhibits spontaneous efflux of catecholamines from perfused dog adrenal are not in accordance with the report of Oset-Gasque et al. [31] that NO *increased* unstimulated efflux of catecholamines from bovine chromaffin cells grown in tissue culture. This may indicate that there are species differences in NO effects between canine and bovine adrenal chromaffin cells. More likely, however, it indicates differences between basal efflux in cultured cells compared to perfused organs which more nearly approximates *in vivo* conditions. As pointed out by Bowers [3] cells in culture are disrupted cells and there are many reported examples of differences in these cells from the *in vivo* situation.

Experimental Protocol.

The present studies were part of a series to determine the effects of NOS inhibition on both basal and evoked effluxes of catecholamines and NPY from dog adrenal gland. In these experiments, stimulated releases separated by rest periods were compared in the presence and absence of L-NMMA in the same gland with L-NMMA being added after 160 min to inhibit NOS. The studies showed that L-NMMA application had clearly different effects on basal and evoked effluxes of these autocoids. It became clear that it was necessary to understand first the effects of L-NMMA on basal effluxes at times which evoked releases were done in the presence of L-NMMA (studies on evoked releases in progress). Basal efflux of catecholamines from dog adrenal gland has previously been studied and shown to be remarkedly stable after the first 60 min of perfusion with a decline occurring at a slow and stable rate for longer than 160 min [10]. Basal efflux does, however, increase during unphysiologic conditions [14] but this did not occur in the present studies under control conditions.

Sources of NO

Possible sources of the NO involved in the regulation of the spontaneous efflux of catecholamines from the adrenal medulla include the preganglionic sympathetic nerves, ganglion cells in the adrenal medulla, the network of intrinsic fibers which extend from the medulla to the subcapsular area of the zona glomulerosa as well as from blood vessels [2,21]. In addition to an effect on catecholamine secretion, NO has been shown to enhance blood flow through the adrenal glands [6], presumably by causing a reduction in resistance to blood flow. In the system used in these studies the flow rate was fixed at 1.5 ml/min. Since rate of flow of the perfusate through the adrenal gland was fixed in our system, an NO-induced effect on flow through the adrenal could not be determined. The NO-induced increase in blood flow through the adrenal [6] should increase clearance of catecholamines from the adrenal gland and entry into the bloodstream. However, it appears from our present study that NO also inhibits basal efflux of E, NE and DA from the chromaffin cells which suggests that during basal conditions increased catecholamine secretion may not always be coupled with increased blood flow through the gland.

Spontaneous efflux of catecholamines from adrenal gland.

The spontaneous efflux of catecholamines that was inhibited by NO in the present study appeared to be an exocytotic release in that this efflux was dependent on the presence of Ca^{2+} in the perfusate. However, we could not demonstrate a difference in the rate of basal efflux of catecholamines from adrenal glands perfused in the presence versus those perfused in the absence of Ca^{2+}, and neuronal NOS is a Ca^{2+}-dependent enzyme [4]. Chritton, et al. [10] also could only clearly demonstrate an effect of Ca^{2+} on basal catecholamine efflux when Ca^{2+}-free perfusates were replaced with Ca^{2+}-replete perfusates in the same experiment i.e. with each adrenal gland acting as its own control. In addition, Chritton et al. [10] found an *increased* basal efflux in the presence of Ca^{2+} i.e. under conditions when NOS could be expected to be more active. This is not consistent with the findings in this study that basal efflux of catecholamines is inhibited by endogenously produced NO. Thus, it is suggested either that, in the absence of Ca^{2+}, intracellular levels of Ca^{2+} were still high enough to maintain a basal activity of NOS or that other neurotransmitter inputs drive

basal efflux of catecholamines from the adrenal gland to a greater extent than does NO. Basal efflux has been shown not to be driven by cholinergic, serotonergic, dopaminergic [9], or opioid [13] stimulation. Basal efflux of catecholamines from dog adrenal glands was, however, increased by atrial natriuretic peptide [12] and it is possible that it is controlled by other as yet unknown transmitter inputs.

The mechanisms underlying basal non-exocytotic efflux of catecholamines from adrenal chromaffin cells as well as that from sympathetic neurons have previously received scant attention. However, there are suggestions that this basal efflux is substantial. As long ago as 1970 [27] it was suggested that the bulk (i.e. up to 70%) of the NE metabolites excreted into urine had their origin not subsequent to exocytotic release from nerve terminals but after intraneuronal metabolism, implying release from vesicles into neuroplasm and from neuroplasm into junctional clefts. Recently, McKinzie et al. [29] showed that, averaged over the entire nervous system, impulse traffic did not account for the major fraction of NE turnover *in vivo* in the rat. The mechanisms underlying such nonexocytotic, Ca^{2+}-independent efflux have been discussed by Adam-Vizi [1]. She suggested that release apparently independent of external Ca^{2+} could be triggered by mobilization of internal Ca^{2+} stores. Alternatively, efflux might be mediated by the NE uptake transporter operating in an inside-outside direction. Although this would be possible in peripheral sympathetic neurons, it would be less likely in adrenal chromaffin cells where a catecholamine uptake mechanism has not been demonstrated unequivocally *in vivo*.

The functional relevance of basal efflux has also received little attention. However, Adam-Vizi [1] points out that a continuous release of transmitters from neurons in a Ca^{2+}-independent manner may be important in maintaining the sensitivity and proper trophic function of the postsynaptic region. It may be that a continuous efflux of hormones from the adrenal medulla has a similar significance for target organs. In addition, a Ca^{2+}-independent release could have pathophysiological importance when Ca^{2+}-independent release of certain transmitter compounds is either a consequence of or a reason for some functionally impaired status [1].

Significance of DOPEG and DOPAC in perfusates.

Considerable quantities of DOPEG and DOPAC are present in plasma of man and experimental animals [18], some of which undoubtedly originates in the central nervous system [24]. DOPEG in plasma has, however, also been shown to be produced in peripheral sympathetic neurons [22], but the formation of DOPEG and DOPAC by the adrenal medulla has previously received little attention [8]. Under conditions of spontaneous efflux, DOPEG production in peripheral sympathetic neurons has been proposed to originate by the action of monoamine oxidase (MAO) subsequent to translocation of NE from vesicles into cytoplasm. Thus basal efflux of DOPEG is unaffected by the presence of inhibitors of neuronal uptake of NE [22]. During nerve stimulation, the efflux of DOPEG increases and the increases are blocked by inhibitors of NE reuptake [34]. Thus, in peripheral sympathetic neurons DOPEG is formed subsequent to two distinct processes: vesicular-cytoplasmic translocation of NE and neuronal reuptake of exocytotically-released NE. DOPEG has previously been shown to be produced in canine adrenal gland, but cocaine, an inhibitor of neuronal reuptake of NE, was without effect on DOPEG or DOPAC efflux under conditions of either spontaneous or evoked efflux [8]. This is compatible with the recent finding [40] that there is no uptake process for catecholamines in intact adrenal gland analogous to neuronal reuptake of released NE in peripheral sympathetic neurons which would be expected in view of the endocrine function of EPI released from the adrenal medulla.

Thus, it appears that DOPEG in adrenal medulla originates solely subsequent to vesicular-cytoplasmic translocation of NE and EPI. The lack of inhibition of NOS or of most NO donors on DOPEG or on DOPAC effluxes indicates that vesicular-cytoplasmic translocation of NE, EPI or of DA is not affected by NO. However, basal efflux of DOPEG and of DOPAC was increased in a dose-dependent manner by the NO donor SNP. This action is difficult to explain in view of the findings that the other NO donor SIN-1 had no effect on DOPEG or DOPAC but it is possible that it is not attributable to NO itself but rather to some other metabolite of SNP causing increased vesicular-cytoplasmic translocation of NE. However, in the presence of L-NMMA the efflux of DOPAC decreased significantly with time and this significant decrease did not occur in the control perfusions. This adds additional evidence to suggest that NO may have a minor effect to increase

translocation of catecholamines from storage vesicles to neuroplasm. Alternatively, these effects would be explained if NO had a minor stimulatory effect on MAO activity.

NO and the efflux of ME from adrenal gland.

ME is one of several neuropeptides previously demonstrated to be present within or released from the adrenal gland in several species [15]. The peptides include ME, neuropeptide Y (NPY), and vasoactive intestinal polypeptide (VIP). Initially we intended to evaluate the effects of NO on the basal efflux of all of these neuropeptides at the same time as those of the catecholamines. However, early studies showed that NPY and VIP were barely measurable in spontaneous efflux from the adrenal glands so studies of these two peptides were not pursued. However, both free ME and TME could be reliably measured in the perfusates. The amounts of TME and free ME in perfusates were very similar. The amounts of TME and ME were also approximately equivalent in adrenal vein in cats under basal conditions [16].

Although inhibition of NO synthesis by the addition of L-NMMA increased the efflux of NE and EPI it was without effect on the efflux of either the free or the extended forms of ME. This may result because the opioids are present not only in the chromaffin granules but also in the terminals of the splanchnic nerve [42] and release from these two sites are affected differently by NO. Gaumann et al. [17] also showed that ME and the catecholamines were not released in proportional amounts during splanchnic nerve stimulation in cats and concluded that releases of the two classes of compounds were under separate control.

Although NPY effluxes from dog adrenal gland during nicotinic or muscarinic stimulation, it did not efflux in significant amounts under basal conditions in the present experiments. This might be expected since the bulk, i.e. approximately 75% of basal efflux from dog adrenal gland, is Ca^{2+}-independent and presumably nonexocytotic [10]. Haass et al. [19] have shown that, in guinea pig heart, NPY is released only during exocytosis, and a differentiation can be made between exocytotic and nonexocytotic releases on the basis of NPY release. However both ME and TME did efflux from the adrenal gland under basal conditions which indicates differences in basal effluxes of peptides either between peptides, between peripheral nerves and the adrenal gland or between

species.

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LEGENDS

Fig. 1: The effect of N^G-monomethyl-L-arginine (L-NMMA; 3 x 10^{-4}M) on the basal efflux of epinephrine (EPI) from dog adrenal gland. In Figs. 1-3 L-NMMA, when added to the perfusate, was present in intervals 2-5. Ca^{2+} was present in all perfusates. Data are the means ±SEM of 5 to 9 experiments. Three L-NMMA experiments were done with L-arginine (10^{-3}M) present. The amounts in perfusates collected per min in intervals 2-5 are expressed relative to those in interval 1. ☐ , control; ■ , L-NMMA; ▨ , L-NMMA and L-arginine. * p <0.05, significant change over time from values measured in interval 1. † $p < 0.05$, significant increase from the value in control perfusates in the same interval. In Figures 1-4, intervals 2 , 3, and 5 are 10-min each and interval 4 is 30-min.

Fig. 2: The effect of N^G-monomethyl-L-arginine (L-NMMA; 3 x 10^{-4}M) on the basal efflux of norepinephrine (NE) from dog adrenal glands. See legend to Fig. 1.

Fig. 3: The effect of N^G-monomethyl-L-arginine (L-NMMA; 3 x 10^{-4}M) on the basal efflux of dopamine (DA) from dog adrenal glands. See legend to Fig. 1.

Fig. 4: The effect of 3-morpholinosydnonimine (SIN-1) on the basal efflux of dopamine (DA) from dog adrenal glands. SIN-1, when added to the perfusate, was present in interval two. Ca^{2+} was present in all perfusates. Data are the means ±SEM of 5 to 9 experiments. The amounts in perfusate collected per min in intervals 2-5 are expressed relative to those in interval 1. * $p < 0.05$, significantly different from the value in controls in the same interval. ☐ , control; ▨ , 10^{-7}M SIN-1; and ▩ , 10^{-5}M SIN-1.

Figure 1

Figure 2

Figure 3

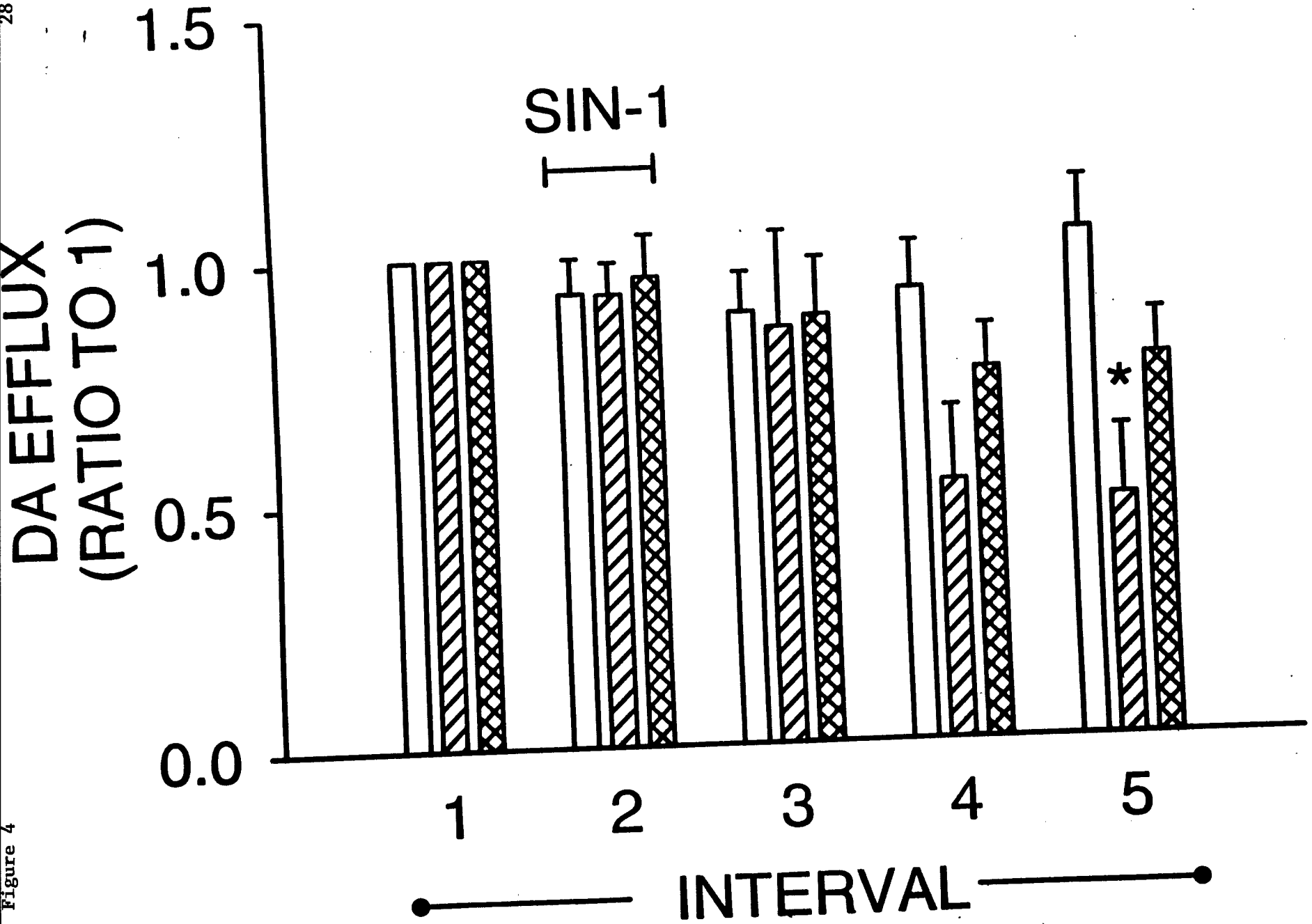

Figure 4

Table 1

The effect of N^G-monomethyl-L-arginine (L-NMMA; 3 x 10^{-4}M) on catecholamine efflux from perfused dog adrenal glands in the absence of Ca^{2+}.

	Interval				
Catecholamine	1	2	3	4	5
EPI					
CONTROL	100	93.6 ± 2.1	84.3 ± 4.5	74.8 ± 8.6*	70.5 ± 12.5*
L-NMMA	100	93.7 ± 5.4	98.7 ± 6.1	103.8 ± 9.1	87.2 ± 12.0
NE					
CONTROL	100	96.1 ± 3.2	86.2 ± 4.8	79.5 ± 8.2	77.1 ± 12.8
L-NMMA	100	96.5 ± 5.4	101.6 ± 7.8	110.2 ± 11.4	94.4 ± 14.9
DA					
CONTROL	100	96.1 ± 3.7	91.4 ± 8.0	80.0 ± 22.7	80.4 ± 41.2
L-NMMA	100	112.5 ± 11.5	107.9 ± 12.2	111.7 ± 11.9	92.0 ± 10.0

The amounts in perfusate collected in intervals 2-5 are expressed relative to those in interval 1. Ca^{2+} was absent from the perfusates throughout the experiments. Data are the means ±SEM of 4 to 6 experiments, * $p < 0.05$ significant decrease over time from corresponding value in interval 1. 1, 2, 3 and 5 are 10 min each and interval 4 is 30 min. EPI: epinephrine, NE: norepinephrine and DA: dopamine.

Table 2

The effect of sodium nitroprusside on catecholamine efflux from perfused dog adrenal glands.

Catecholamine	Control	10^{-6}M SNP	10^{-4}M SNP
Interval 2			
EPI	95.2 ± 3.2	78.6 ± 2.5*	118.5 ± 23.9
NE	94.2 ± 3.6	81.2 ± 4.6*	127.7 ± 20.4
DA	92.6 ± 7.2	79.9 ± 5.3	159.2 ± 31.9
Interval 3			
EPI	86.3 ± 4.2	84.3 ± 2.5	113.4 ± 11.9
NE	85.8 ± 4.5	82.8 ± 4.0	112.2 ± 14.9
DA	88.0 ± 8.2	100.1 ± 12.8	132.5 ± 27.3

The amounts of the compounds in perfusates collected in intervals 2 and 3 are expressed relative to those in interval 1. Ca^{2+} was present in the perfusates throughout the experiments. Data are the means ±SEM of 5 to 7 experiments, * $p < 0.05$, significantly different from the control values in the same interval. EPI: epinephrine, NE: norepinephrine, DA: dopamine and SNP: sodium nitroprusside,

APPENDIX

PROJECT 3

"Effects of Xl-Hgb Solution on Local and Baroreflex Control of Resistance"

Dr. M.J. Joyner

Local cholinergic mechanisms mediate nitric oxide-dependent flow-induced vasorelaxation in vitro

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Martin, Creston M., Abel Beltran-del-Rio, Alison Albrecht, Robert R. Lorenz, and Michael J. Joyner. Local cholinergic mechanisms mediate nitric oxide-dependent flow-induced vasorelaxation in vitro. *Am. J. Physiol.* 270 (*Heart Circ. Physiol.* 39): H442–H446, 1996.—To determine whether local cholinergic mechanisms evoke nitric oxide (NO)-mediated flow-induced vasorelaxation, canine coronary artery rings without endothelium were suspended beneath an organ chamber that contained a stainless steel tube and a femoral artery segment with endothelium. The rings were superfused at a basal rate of 1 ml/min with physiological salt solution that was bubbled with 95% O_2-5% CO_2 and maintained at 37°C. They were stretched to optimal length and contracted with prostaglandin $F_{2\alpha}$ (2×10^{-6} M). When flow through the stainless steel tube (direct superfusion) was increased from the basal rate of 1 to 4 ml/min, coronary force did not change. Superfusion of the rings ($n = 8$) with effluent from the femoral segment (endothelial superfusion) at 4 ml/min to study flow-induced vasodilation caused a 67.3 ± 10.8% relaxation. Treatment of the segment with the NO synthase blocker N^G-monomethyl-L-arginine (10^{-4} M) eliminated the relaxation seen during endothelial superfusion ($P < 0.05$ vs. control). Application of atropine (10^{-6} M) to additional femoral segments ($n = 8$) abolished the coronary relaxation observed during endothelial superfusion at 1 ml/min, and the flow-induced relaxation observed at 4 ml/min was reduced from 64 ± 8.3 to 27 ± 5.6% ($P < 0.05$ vs. control). In studies on additional segments and rings ($n = 6$), the flow-induced relaxations at 4 ml/min of endothelial superfusion were blunted from 86 ± 10 to 28 ± 13% after the segments were treated with acetylcholinesterase (0.00028 U/min for 20 min). These data indicate that basal- and flow-induced release of NO from the vascular endothelium can be mediated by local cholinergic mechanisms. It is possible that flow causes acetylcholine release from certain endothelial cells, which stimulates NO release from these cells or from neighboring endothelial cells.

vasodilation; flow-induced dilation; acetylcholine

INCREASING FLOW through blood vessels with intact vascular endothelium evokes release of nitric oxide (NO) and causes "flow-induced vasodilatation" (2, 14, 17). The mechanisms responsible for this NO release are obscure. The current concept is that mechanical interactions between fluid flowing in the lumen of the blood vessel and the vascular endothelium cause the activation of K^+ channels and subsequent NO release (5, 11).

The vascular endothelium also possesses muscarinic receptors that, when stimulated, cause the release of NO (7, 16). However, the physiological function of these receptors is unclear (17). There is some evidence that acetylcholine (ACh) from autonomic nerves can reach the vascular endothelium and evoke the release of NO (1). In addition, certain endothelial cells may be able to synthesize ACh, and recent evidence from cultured endothelial cells suggests that flow can induce the release of ACh (9, 10, 12). In humans, the profound forearm vasodilation seen during mental stress might be explained by local cholinergic mechanisms operating to cause NO release (6). However, no studies to date have attempted to determine whether locally released ACh might play a functionally significant role in mediating NO release from the vascular endothelium. With this information as a background, we tested the hypothesis that local cholinergic mechanisms can cause flow-induced release of NO and subsequent vasorelaxation using a standard bioassay system to study flow-induced vasorelaxation (14). Our results provide physiological evidence that local ACh release may be an important mediator of flow-mediated NO release from the vascular endothelium.

METHODS AND PROCEDURES

General Methods

Femoral and left circumflex coronary arteries were obtained from dogs ($n = 31$) anesthetized with pentobarbital sodium and killed by exsanguination following a protocol approved by the Institutional Animal Care and Use Committee. A bioassay system similar to that described by Rubanyi and colleagues (14) was used. A coronary artery ring in which the endothelium had been removed by gently rubbing the lumen with a small forceps (7, 17) was suspended beneath an organ chamber that contained both a stainless steel tube and a femoral artery segment with endothelium (Fig. 1). The femoral artery segment and stainless steel tube were perfused with physiological salt solution (PSS) of the following composition (in mM): 110.5 NaCl, 25.7 $NaHCO_3$, 5.6 dextrose, 3.4 KCl, 2.4 $CaCl_2$, 1.2 KH_2PO_4, and 0.8 $MgSO_4$. The PSS contained indomethacin (10^{-5} M) to inhibit cyclooxygenase and L-arginine (10^{-6} M) to provide substrate for the synthesis of NO (13).

The ring was superfused with PSS passed through the stainless steel tube for ~45 min. During this interval, the rings were stretched in a stepwise manner using the standard technique until basal tension reached ~10 *g*, the optimal tension for isolated coronary artery rings to contract (4, 15). Then prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$; 2×10^{-6} M) was added to the PSS. When the contraction had reached a steady state, ACh was infused into the direct superfusion line by means of an infusion pump at a rate of 0.1 ml/min to reach 10^{-6} M final perfusate concentration. Absence of functional endothelium was confirmed by failure of the rings to relax when exposed to ACh.

The system was bubbled with 95% O_2-5% CO_2 and maintained at 37°C. It was designed so that drugs could be administered selectively to either the femoral segment or

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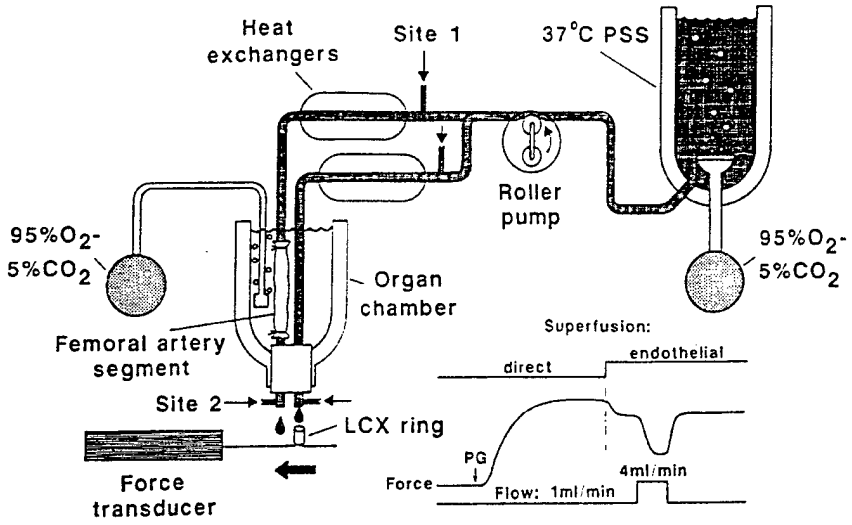

Fig. 1. Schematic representation of experimental design. A femoral artery segment with endothelium is mounted in an organ chamber containing physiological salt solution (PSS) maintained at 37°C and bubbled with 95% O_2-5% CO_2. Hydrostatic pressure of fluid surrounding femoral segment causes partial collapse of this vessel. Chamber also contains a stainless steel tube. Further details of apparatus are described in METHODS AND PROCEDURES. When position of the left circumflex coronary ring (LCX) is shifted so that it is exposed to the effluent from the femoral segment at 1 ml/min, there is a highly variable relaxation of the coronary ring, indicating basal release of a relaxing factor. Flow through segment is then increased to 4 ml/min while force in the coronary ring is continuously recorded. Drugs can be given selectively to either the femoral segment (*site 1*) or to the coronary ring (*site 2*). In our protocols with atropine and acetylcholinesterase (AChE), we used 2 complete sets of the bioassay system so that experiments could be conducted in parallel with treated and time control vessels.

coronary ring. The flow rate through the stainless steel tube or the femoral artery segment was varied during contraction to $PGF_{2\alpha}$, and the effects on force in the coronary ring were observed. In preliminary studies, changes in the basal flow rate from 1 to 4 ml/min through the stainless steel tube had no effect on force in the coronary rings.

In some experiments, two identical systems were used so that parallel studies on paired sets of blood vessels obtained from the same donor animal could be conducted. When parallel studies were performed, one set of the paired vessels served as controls while the other set was subjected to the pharmacological interventions. During the paired studies, one of the coronary rings usually showed greater and more consistent relaxations during several preliminary runs of endothelial superfusion at 1 and 4 ml/min. This ring was always selected for subsequent drug treatment. To ensure that this selection process did not qualitatively bias our results, at the end of each experiment we also applied the drugs under study to the "control" set of blood vessels. The control vessels always responded in a manner similar to that seen in the "experimental" set of vessels.

Drugs

All drugs were prepared fresh daily. ACh, acetylcholinesterase (AChE), atropine, bradykinin, indomethacin, L-arginine, and $PGF_{2\alpha}$ were obtained from Sigma. N^G-monomethyl-L-arginine (L-NMMA) was obtained from Calbiochem.

Protocols

Table 1 summarizes the nature of the specific protocol and the number of donor animals and vessels used in each protocol.

Effects of L-NMMA treatment of femoral segment on coronary responses to altered flow. In this protocol, femoral segments with endothelium and coronary rings without endothelium were mounted and precontracted with $PGF_{2\alpha}$ as described above. Eight segments and rings were studied from five donor animals. The coronary ring was then superfused directly with PSS at 1 ml/min. The coronary ring was then quickly positioned so that it was superfused with effluent from the femoral segment at a basal rate of 1 ml/min. This caused relaxation of the coronary ring. When force stabilized after several minutes, flow through the femoral segment was increased to 4 ml/min to evoke a flow-induced relaxation. When the flow-induced coronary relaxations to superfusion at 4 ml/min had stabilized, the coronary ring was returned to direct superperfusion at 1 ml/min. The tension in the coronary ring then returned to its original level. After stabilization of the force, this procedure was repeated. The femoral segment was then treated with the NO synthase blocker L-NMMA (10^{-4} M) for 20 min. This concentration was selected on the basis of its inhibitory effect on endothelium-dependent relaxations induced by ACh in vascular smooth muscle (13). This was done during direct superfusion of the coronary ring so that no L-NMMA reached the coronary ring. After treatment of the femoral segment with L-NMMA, the effects of endothelial superfusion at 1 and 4 ml/min on tension in the coronary ring were again examined. The purpose of this

Table 1. *Summary of experimental interventions*

Treatment	Site	Paired Study	No. Donor Animals	No. of Segments and Rings	Bradykinin to Segment
L-NMMA	Femoral segment	No	5	8	
Atropine	Femoral segment	Yes	8	16	
	Coronary ring	No	6	6	Yes
	Femoral segment	Yes	6	12	Yes
AChE	Femoral segment	Yes	6	12	Yes

Total no. of donor animals and no. of segments and rings are 31 and 54, respectively. L-NMMA, N^G-monomethyl-L-arginine; AChE, acetylcholinesterase.

protocol was to demonstrate that the basal and flow-induced relaxations observed in the coronary ring during superfusion via the femoral artery at 1 and 4 ml/min were caused by NO release from the femoral endothelium.

Effects of atropine treatment of femoral segments on coronary responses to altered flow. Similar experiments were performed with tissues from eight animals using two parallel sets of the experimental system shown in Fig. 1. One system served as a control. In the other, the femoral segment was treated with atropine (10^{-6} M) for 20 min after demonstration of successive basal and flow-induced relaxations during endothelial superfusion at 1 and 4 ml/min. This concentration of atropine blocks the effects of ACh on isolated endothelial cells (2). In vessels from six additional animals (Table 1) atropine was applied to coronary artery rings and not the segments to determine whether it blunted the relaxations seen during endothelial superfusion at 1 or 4 ml/min. In paired studies on vessels from six more animals, bradykinin (10^{-7} M) was also used to evoke a nonmuscarinic receptor-mediated release of NO from the vascular endothelium before and after the femoral segments had been treated with atropine. The purpose of these control studies was to ensure that the atropine did not cause some general inhibition of NO release in femoral segments (3, 8).

Effects of AChE treatment of femoral segments on coronary responses to altered flow. In a third series of studies performed with tissues from six additional animals using two parallel experimental setups, AChE (0.00028 U) from the Amazonian electric eel (*Electrophorus electricus*) was applied to one of the femoral artery segments for 20 min after the coronary responses to endothelial superfusion had been observed (see Fig. 3). This dose was selected on the basis of pilot studies showing it did not cause any direct effects on the force in an isolated coronary ring. Additionally, after the AChE trials, bradykinin (10^{-7} M) was administered to the femoral artery segments to evoke a non-ACh-dependent receptor-mediated release of NO (3, 8). Bradykinin was used to evaluate whether the AChE had some nonspecific effects on the isolated blood vessels.

Statistics

In all experiments conducted, the responses from the same vessels were compared before and after the various interventions. The effects of the interventions were evaluated using paired *t*-tests. Significance was set at the 0.05 level for all comparisons. Values are reported as means ± SE.

RESULTS

Effects of L-NMMA Treatment of Femoral Segment on Coronary Responses to Altered Flow

In experiments on eight sets of vessels from five animals, endothelial superfusion at 1 ml/min caused a variable (~10–40%) relaxation of the coronary rings that had been precontracted with $PGF_{2\alpha}$. When femoral flow was increased to 4 ml/min there was a 67 ± 10.8% (n = 8) flow-induced relaxation from the baseline value observed during direct superfusion. Treatment of the femoral segments with L-NMMA (10^{-4} M) to block NO synthase abolished relaxations during endothelial superfusion at 1 ml/min. After L-NMMA, an increase in flow to 4 ml/min caused a small 5 ± 5.3% increase in coronary force ($P < 0.05$ vs. control).

Effects of Atropine Treatment of Femoral Segment on Coronary Responses to Altered Flow

Before atropine treatment, endothelial superfusion of the experimental vessels at 1 ml/min caused a mean reduction in coronary force of 22 ± 11.1% observed (n = 8). When flow was increased to 4 ml/min, the mean fall in force was 64 ± 8.3% (n = 8) from the baseline value observed during direct superfusion. Atropine treatment of the femoral artery segment before endothelial superfusion of the coronary rings caused a barely detectable (3 ± 4.7%) contraction of the coronary rings when endothelial superfusion at 1 ml/min was initiated. During endothelial superfusion at 4 ml/min, a flow-induced relaxation of only 27 ± 5.6% was observed ($P <$ 0.05 vs. control). In the rings superfused by the untreated segments (n = 8) total relaxations of 43 ± 13.8 and 50 ± 8.9% were observed during successive increases in flow to 4 ml/min. Figure 2 is an individual record from an experiment showing the effects of atropine treatment of a femoral segment on flow-induced relaxations in a coronary ring.

In the vessels where atropine was applied selectively to coronary artery rings (n = 6) it did not blunt the basal or flow-induced relaxations seen with endothelial superfusion at 1 or 4 ml/min. In further control experiments (n = 6), bradykinin (10^{-7} M) applied to femoral artery segments caused a 75 ± 9% relaxation of coronary rings before atropine treatment of the segments and a 67 ± 10% relaxation after atropine. Successive administration of bradykinin to the paired untreated femoral vessels (n = 6) caused similar relaxations.

Effects of AChE Treatment of Femoral Segment on Coronary Responses to Altered Flow

Before AChE, endothelial superfusion at 1 ml/min caused a 13 ± 5.5% relaxation of the coronary rings (n = 6). Increases in flow to 4 ml/min caused an 86 ± 10.0% relaxation in the coronary rings from the baseline value observed during direct superfusion at 1 ml/min. After AChE, endothelial superfusion at 1 ml/min caused a slight contraction (5 ± 2.1%) of the coronary rings, and an increase in flow to 4 ml/min caused a relaxation of only 28 ± 13% ($P < 0.05$ vs. control). At a femoral flow of 4 ml/min, the relaxations seen in the control vessels averaged 44 ± 8.2 and 52 ± 16.0% during repeated runs. Figure 3 is a representative tracing from a study where AChE was applied to the femoral artery segment. Administration of bradykinin to the femoral artery segments after AChE caused a 67 ± 10.4% relaxation of the coronary rings.

DISCUSSION

The major finding of this study is that local cholinergic mechanisms can mediate NO-dependent flow-induced vasorelaxation. This finding is the first demonstration that such mechanisms are potentially important physiological regulators of NO release from the vascular endothelium. There are several observations that support this conclusion. First, treatment of

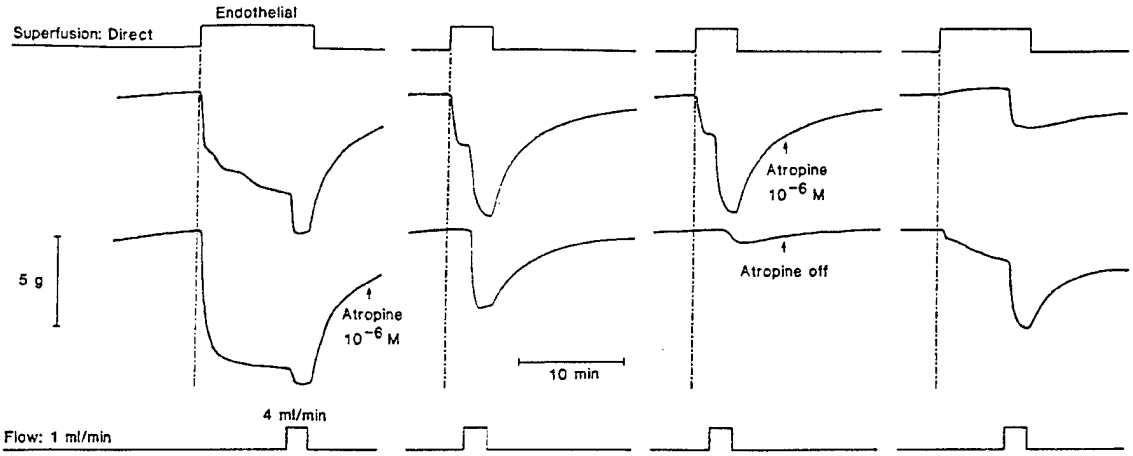

Fig. 2. Individual record showing effects of 4 successive runs of endothelial superfusion at 1 and 4 ml/min on force in contracted coronary artery rings. Experiments were conducted in parallel using 2 sets of the bioassay system described in Fig. 1. Both coronary rings relaxed markedly during endothelial superfusion at 1 ml/min, with a small further relaxation seen when flow through femoral segment was increased to 4 ml/min. During second run in control (*top*) ring, there was less relaxation during endothelial superfusion at 1 ml/min than in first run, but a marked fall in force was observed when flow was increased to 4 ml/min. In ring superfused by atropine-treated segment, relaxation during endothelial superfusion at 1 ml/min was abolished, and total fall in force at 4 ml/min was reduced. During the third run, responses were maintained in control (*top*) vessels at 1 and 4 ml/min of endothelial superfusion, but relaxation was nearly absent in ring superfused by femoral segment treated with atropine. Before the fourth run, the control (*top*) femoral segment was treated with atropine, and coronary relaxations to femoral superfusion at 1 and 4 ml/min were blunted. By contrast, relaxations in experimental (*bottom*) ring returned during the fourth run as atropine washed out of the femoral segment.

the femoral segments with L-NMMA blocked the basal relaxations seen during endothelial superfusion at 1 ml/min and the flow-induced relaxations observed at 4 ml/min. This observation confirms that the mechanism primarily responsible for this vasorelaxation is NO (5). Additional evidence that the NO was released from endothelial cells and not from other constituents of the vessel wall was demonstrated in a previous study, using the same bioassay system that showed no coronary relaxation when flow through a femoral artery segment without endothelium was increased (14). Second, during pilot studies, no relaxation of the coronary ring was seen during direct superfusion at various rates. Third, the basal- and flow-induced relaxations normally seen during endothelial superfusion at 1 and 4 ml/min were also blunted by treatment of the femoral artery segments with either atropine or AChE. With atropine, the effects of any locally released ACh on muscarinic receptors in endothelial cells should have been blocked. With AChE, any ACh released by endothelial cells in the femoral artery segments should have been degraded and unable to act on the muscarinic receptors. Fourth, the relaxations in the coronary rings evoked by bradykinin administration to the femoral segments were preserved after treatment of the segments with atropine or AChE. This indicates that these observations were not the result of some nonspecific drug effects on the isolated vessels. The key question that remains concerns the source of ACh.

Certain endothelial cells can synthesize ACh, but whether this ACh contributes to the vascular regulation has been unclear (12). Milner and colleagues (10) have presented evidence for ACh release from the vascular endothelium during hypoxic vasodilation in

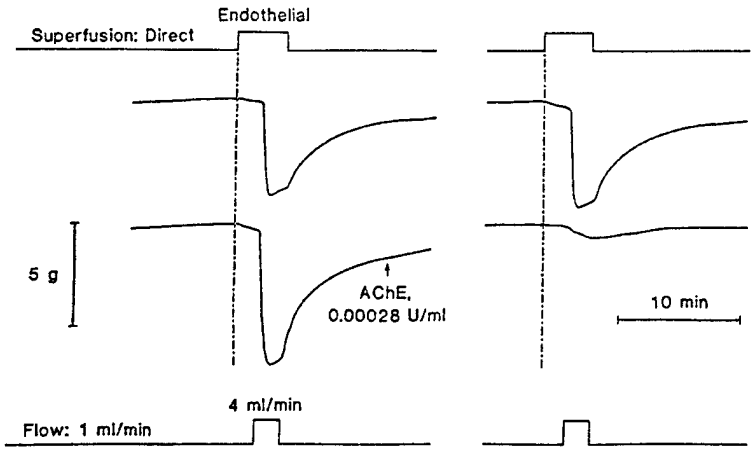

Fig. 3. Individual record showing effects of altered flow through a femoral artery segment treated with AChE on force in contracted coronary artery ring. Experiments were conducted in parallel using 2 sets of the bioassay system described in Fig. 1. Both coronary rings showed a small relaxation with endothelial superfusion, with marked relaxation seen when flow through femoral segment was increased to 4 ml/min. These force responses were consistent during a repeated run in control (*top*) vessel. Treatment of 1 femoral segment with AChE eliminated the coronary relaxation observed at 1 ml/min and blunted relaxation when femoral flow was increased to 4 ml/min.

coronary rings. There have also been demonstrations in cultured endothelial cells, showing that flow can induce the release of ACh (9). These observations suggest that some endothelial cells can synthesize and release ACh in response to alterations in flow through a vessel. If sufficient ACh were synthesized in the endothelial cells it would appear reasonable to suggest that this ACh could be released because of mechanical interactions between fluid flowing in the blood vessel lumen and the vascular endothelium. Such ACh release could then stimulate muscarinic receptors on neighboring cells and evoke NO release (6).

Previous studies on the mechanisms of flow-induced release of NO have suggested that mechanical factors activate an endothelial K^+ channel that stimulates NO release (5, 11). This mechanism could explain NO release from the vascular endothelium that is not initiated by receptor-mediated events. It might also account for the modest coronary relaxations that remained after treatment of femoral segments with atropine or AChE, but not when NO synthase was inhibited by administration of L-NMMA.

There are several aspects of the current study that should be considered before local ACh release can be viewed as a major regulator of NO-mediated flow-induced vasodilation in vivo. First, attempts to identify a large number of ACh-releasing endothelial cells on the basis of histochemical techniques have proven difficult (10). Second, the femoral flow rates used were quite low in comparison to those in vivo (i.e., 50–80 ml/min; Dietz, personal communication). This was necessary because it is difficult to maintain the integrity of a bioassay system at such high flow rates and because the concentration of any substance reaching the bioassay tissue would be quite low with such a high flow of PSS. This would make it extremely difficult to observe an effect of altered flow on tension in the coronary ring of the bioassay system. Third, we studied large-conduit vessels that are anatomically remote from resistance vessels in vivo. Fourth, our bioassay system requires that the substance of interest be released into the vessel lumen. The effects of any substances released abluminally that might be physiologically important were not apparent using the current approach.

In summary, flow-induced vasodilation is an important physiological regulator of vascular smooth muscle tone during a variety of physiological responses. In addition, it is well known that ACh causes relaxation of blood vessels in vivo and in vitro on the basis of endothelial NO release (7, 16). However, the physiological relevance of ACh-mediated NO release has been obscure. Our findings are consistent with the concept that some vascular endothelial cells form ACh and that this ACh participates in flow-induced release of NO (9, 10, 12, 17).

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REVIEW ARTICLE

Blood Substitutes: Fluids, Drugs, or Miracle Solutions?

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By the late 1800s efforts were being made to find a parenteral solution for treating anemia (1–3). Interest in such solutions intensified as a result of World War I, when hypovolemia and hemorrhage became more widely appreciated as causes of "circulatory shock" (4–5). Although no oxygen-carrying, volume-expanding solution was identified, clinical experience suggested that shock was caused by hypovolemia and could be reversed by administration of various fluids intravascularly (6). Subsequent clinical experience and laboratory investigations have confirmed the utility of crystalloids, colloids, and/or blood administration in treating hemorrhagic shock, although the optimal timing of such treatment remains controversial (6–9).

The term "blood substitutes" has been used to broadly describe oxygen-carrying, volume-expanding solutions. However, blood performs a host of functions beyond the transport of oxygen whereas the goal of blood substitutes is to transport oxygen and expand blood volume (10). A more accurate definition for blood substitutes is "oxygen-carrying volume expanders." Oxygen-carrying volume expanders have long been attractive to military medical organizations faced with the logistic constraints of the battlefield. An oxygen-carrying volume expander that is easy to store, transport, and administer could be life-saving for injured soldiers (10,11). Concern over fatal blood-borne pathogens, including hepatitis and human immunodeficiency virus (HIV), also make oxygen-carrying volume expanders attractive for civilian use (10,12).

Blood substitutes may be particularly useful in the next several decades. The United States blood supply faces major challenges associated with an aging population and potentially inadequate rates of volunteer donation by healthy citizens. Approximately 14 million units of whole blood are collected in the United States each year, and there are roughly 12 million transfusions of red blood cells (RBCs) (13–16). Half of these RBCs are transfused to patients 65 yr or older, although this age group represents only 12.5% of the population (Figure 1). It is estimated that by the year 2030, approximately 22% of the population will be elderly (65 yr of age or older), and the absolute number of this group will more than double (17). At present rates of RBC utilization, the elderly population alone will require 12–13 million units of red cells per year by the year 2030 (13). If present donation patterns in the United States remain constant, a shortfall of 4 million units of RBCs is projected in 2030. In this context, a safe and effective blood substitute could be useful to meet this projected shortfall in the red blood cell supply.

A variety of substances that transport oxygen and augment intravascular volume in the absence of red cells are emerging as possible blood substitutes for use in humans. These include hemoglobin solutions, liposome-encapsulated hemoglobin (LEH), and perfluorocarbons (10). None of these compounds replace the coagulant or immunologic functions of blood products, and all have a limited circulatory half-life in comparison to RBC transfusion. Since 60%–70% of RBC transfusions occur in surgical patients during the perioperative period (18), it is important that basic issues related to the emerging field of oxygen-carrying volume expanders be understood by anesthesiologists. The purpose of this review is to discuss issues related to the various oxygen-carrying, volume-expanding solutions by addressing the following questions:

1. What is the need for blood substitutes in clinical transfusion practice?
2. What are the ideal properties of an oxygen-carrying volume expander?
3. What types of oxygen-carrying solutions are under development?
4. What are the potential clinical applications of these products?
5. What is the ultimate definition of their efficacy?

Need for Blood Substitutes

Blood substitutes may be used to address three basic issues in transfusion medicine. First, RBCs can be

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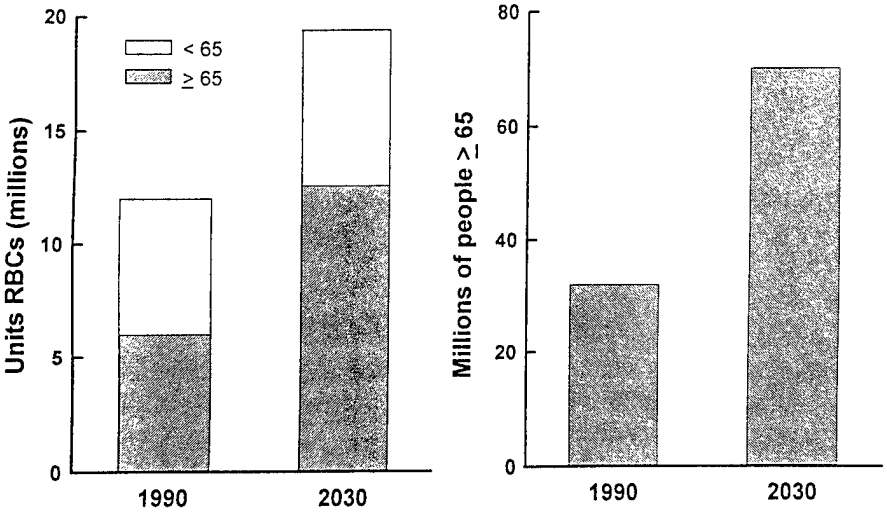

Figure 1. The potential effects of aging on the future demand for red blood cells (RBCs) in the United States. In 1990, approximately 12 million units of RBCs were transfused. Half of these units were transfused to the elderly (age ≥65 yr). In the year 2030, it is estimated that the total demand for RBCs will exceed 19 million units annually, with over 12 million of these units administered to the elderly. [Data from Vamvakas and Taswell 1994 (13).]

difficult to store and transport, and their safe administration requires laboratory screening for donor-recipient compatibility and infectious disease. A product with a long shelf-life that is universally compatible could solve many of these problems. Second, despite rigorous screening for infectious disease, there is modest (1:3000) risk of hepatitis C transmission and a smaller (1:100,000 to 1:1,000,000) risk of HIV transmission per unit transfused (19–24). Blood transfusion can also result in bacterial, parasitic, and other viral disease transmission, but the risk of clinically significant disease from these is low (25). The risk of infectious disease transmission could be further minimized or eliminated with a safe blood substitute. Third, inadequate donation of blood by healthy citizens in the context of an aging population is already causing significant shortages of RBCs in some locales (26). These local shortages could potentially be eliminated by an oxygen-carrying, volume-expanding solution that has a long intravascular half-life. Even if an ideal oxygen-transporting, volume-expanding solution was developed, there will still be a need for blood components, such as platelets, fresh frozen plasma, and cryoprecipitate. This conceivably could lead to a situation where whole blood was collected to harvest these components and the RBCs were subsequently wasted. Increased blood donation by volunteers may be the simplest and most cost-effective solution to the supply problem (13).

Ideal Properties

As a result of a variety of factors, RBC transfusions have never undergone a classic broad-based clinical trial designed to demonstrate their efficacy. Nonetheless, RBC transfusions are widely used and they are the standard to which oxygen-carrying volume-expanding solutions are compared. RBCs are usually administered in a "packed" form that consists of approximately 225 mL volume with a hematocrit of 70%–80% (i.e., 25 g/dL of hemoglobin) (27). Seventy percent of the red cells in a unit of packed RBCs will survive in the circulation for more than 24 h after transfusion (27). The surviving cells are then assumed to have a life-span similar to native blood (i.e., weeks to months). Important properties of RBCs include: 1) high oxygen-carrying capacity; 2) ability to transport oxygen when oxygen tension is in the normal physiologic range; 3) desirable elimination characteristics; and 4) low incidence of side effects when appropriately screened and administered. Undesirable properties of RBCs include: 1) relatively short shelf-life (i.e., weeks) in most forms; 2) antigenicity requiring pretransfusion testing of donors and recipients; 3) transfusion reactions; 4) dependence on a limited donor pool; 5) infectious disease transmission; and 6) suppression of the normal immune system (10,28,29).

Although the oxygen-carrying volume expanders under development have few of the undesirable characteristics associated with RBCs, none has all of the desirable properties. With most products currently being developed, there are concerns related to short intravascular half-lives, routes of elimination, physiologic side effects (e.g., hypertension), and interactions with coexisting diseases. As blood substitutes emerge it should be emphasized that the current blood supply is safe in comparison to many forms of medical or industrial technologies. For example, the risk of dying in a fatal automobile accident in the United States is approximately 0.002% annually (30). The overall yearly risk of dying from an allogeneic blood transfusion is several orders of magnitude less at approximately 0.0001% or lower (19,22,27). Therefore, the overall safety of any RBC substitute will have to be very high to be as safe as allogeneic blood. In addition, the cost of these solutions will also have to compare

favorably with that of RBCs. It is currently estimated that the hospital acquisition cost of one unit of RBCs is approximately $52–$64 (31,32).

Types of Oxygen-Carrying Volume Expanders

Several types of products are under development as potential oxygen-carrying volume expanders (12). These products are classified in one of three categories: 1) hemoglobin solutions, containing some modification of the hemoglobin molecule; 2) liposome-encapsulated hemoglobin solutions, containing hemoglobin within a synthetic membrane; 3) perfluorocarbons, organic solutions with high oxygen solubility.

Hemoglobin Solutions

In the 1930s Amberson began the first systematic investigation of an intravenous bovine hemoglobin-in-saline solution as an oxygen-carrying volume expander in several animal species (33). Animals survived for up to 36 h after exchange transfusions, with death due to hemoglobin loss from the circulation. In the initial hours after the exchange transfusions, oxygen consumption remained normal and arterial hypertension was observed. Subsequently, Amberson et al. (34) used a human hemoglobin-in-saline solution a total of 77 times in 14 patients with anemia. Results included restoration of blood volume, increased oxygen-carrying capacity, and stimulation of hematopoiesis as evidenced by an increased reticulocyte count. When a modest volume was given to a patient suffering from acute hemorrhagic shock as a result of obstetrical bleeding, prompt restoration of arterial blood pressure was observed. Side effects reported in these patients included renal dysfunction and an acute increase in mean arterial pressure above normal values.

When hemoglobin is liberated from human RBCs, its tetrameric structure of two α and two β chains dissociates into dimers consisting of α and β hemoglobin chains or hemoglobin monomers (35). This dissociation decreases the molecular weight from approximately 64 kd per tetramer to 32 kd per dimer or 16 kd per monomer. With this smaller size, the dimer is filtered by the kidneys, reducing its intravascular retention time (36–38). Additionally, the oxygen-binding cooperativity of hemoglobin in this dimeric form is lost, 2,3-diphosphoglycerate (2,3-DPG) is no longer a major regulator of the oxygen-hemoglobin dissociation curve, and the curve is shifted to the left (38). This left shift causes P_{50} to decrease to a Po_2 of 12–16 mm Hg and limits oxygen unloading at the tissues (38).

Renal damage occurs when filtered hemoglobin precipitates in the acidic ascending limb of the loop of Henle (36,39). Red cell stromal debris remaining in the hemoglobin solution as a result of inadequate purification may also contribute to renal damage and systemic toxicity due to activation of the complement cascade (40,41). Removal of red blood cell stroma from hemoglobin solutions reduces these side effects (41,42).

Four general classes of stroma-free hemoglobin solutions are under development at present. They are 1) intramolecularly cross-linked hemoglobin, 2) polymerized hemoglobin, 3) conjugated hemoglobin, and 4) the newly emerging hemoglobin microbubbles (43–47). All four solutions contain a modified hemoglobin molecule. These modifications are used to increase molecular size and decrease renal filtration, prolong intravascular persistence, and to ensure a normal P_{50} of hemoglobin. Figure 2 is a schematic representation of some concepts related to hemoglobin solutions currently under development.

With cross-linked hemoglobin, the tetrameric structure of hemoglobin is maintained by an intramolecular cross-link between the α or β hemoglobin chains. For example, when two α chains are cross-linked, the two β chains remain associated with them on the basis of weak chemical forces (35,48). Several cross-linking strategies are now under commercial development. These include creation of chemical bonds between hemoglobin chains (12,49–52) and use of genetic engineering to create one or more molecular bridges between two α chains that are synthesized in microorganisms (53–55). Polymerized hemoglobin solutions contain either oligomers of cross-linked hemoglobin or polymers of hemoglobin chains (45,56–58). Conjugated hemoglobin is formed by linking free hemoglobin to soluble nonhemoglobin polymers (59–62). In one strategy currently under development, bovine hemoglobin is conjugated with polyethylene glycol (46,63). Solutions containing cross-linked hemoglobin, polymerized hemoglobin, and conjugated hemoglobin sustain life during exchange transfusions that eliminate almost all of the experimental animals native RBCs (44–46). Several of these solutions have undergone extensive animal testing and are entering early safety trials in humans.

Hemoglobin microspheres are a much more recent development that shows promise (47). This technology uses high-intensity ultrasound to form microbubbles which have shells consisting of approximately one million hemoglobin molecules which are chemically cross-linked by superoxide formed during the sonication process. Characterization of these microspheres shows an oxygen-carrying capacity (0.32 mL of O_2/mL solution) greater than that of blood, oxygen affinities similar to those of native hemoglobin, and minimal degradation in solution after storage for 6 mo at 4°C. Development of hemoglobin microbubbles is

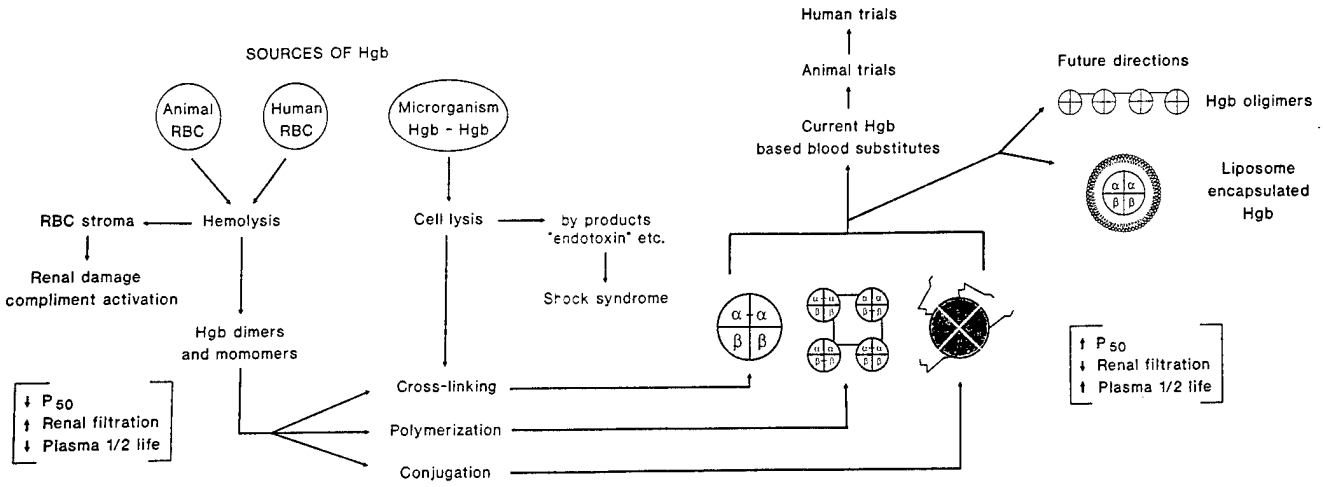

Figure 2. Schematic representation of hemoglobin-based blood substitutes now under development. Animal, human, or genetically engineered (microorganism produced) sources of hemoglobin are used. The hemoglobin is then separated from either the red blood cells or microorganism. If the source of hemoglobin is animal or human, hemoglobin dimers and monomers result. These dimers and monomers are associated with a reduced P_{50}, renal filtration, and a very short plasma half-life. To stabilize the smaller hemoglobin units obtained from animal or human red cells, these hemoglobin dimers and monomers are modified by either cross-linking, polymerization, or conjugation. The hemoglobin obtained from microorganisms is cross-linked during the synthetic process in the microorganism. The resultant cross-linked, polymerized, or conjugated hemoglobins have P_{50} values in the physiologic range, are subject to significantly less renal filtration, and also have a prolonged plasma half-life (h). Products based on these schemes represent the currently emerging hemoglobin-based blood substitutes. Such products are undergoing testing in animals and some are nearing trials in humans. In the future it is likely that various combinations of hemoglobin tetramers (known as oligomers) or liposome-encapsulated hemoglobin will be developed. One potentially attractive feature of such products would be a dramatically increased plasma half-life. The newly developed hemoglobin microbubbles (not represented) are an example of one of these products. Although not tested yet in animals or humans, the microbubbles theoretically will have an increased intravascular retention time due to their larger size (one-half that of an erythrocyte). Each of the basic forms of hemoglobin-based blood substitutes has been demonstrated to transport oxygen, sustain life during severe anemia, and be effective as a volume resuscitation fluid. However, it is unclear whether such products can be used in humans in a way that decreases the use of blood products, or significantly alters transfusion-related morbidity and mortality. Hgb = hemoglobin; RBC = red blood cells. (For discussion of issues related to the ultimate efficacy of blood substitutes, please see Figure 6).

in very early stages compared to the other types of hemoglobin solutions, and no animal or human studies have yet been conducted.

Sources of Hemoglobin. If hemoglobin solutions were used to replace the oxygen-carrying capacity of 10% of the packed RBCs transfused annually in the United States, 60,000–70,000 kg of hemoglobin would be required each year (19,64). Hemoglobin-based blood substitutes currently under development use human, animal, and biotechnologic sources of hemoglobin. There are potential logistic and safety problems associated with each of these sources.

Several products under development use human hemoglobin derived from outdated banked blood (52,65,66). With a shrinking donor pool in the United States and better inventory control in most blood banks, fewer units are becoming outdated. Therefore, it seems unlikely that outdated banked blood could provide enough hemoglobin to replace 10% of that volume now provided by RBCs unless there was substantial recruitment of new donors. A greater pool of hemoglobin might be available if it was liberated from donor RBCs as a processing step in blood component harvest from whole blood. In this concept, the hemoglobin in red cells would be viewed as a separate component of whole blood similar to platelets, fresh frozen plasma, cryoprecipitate, and albumin. This strategy would allow a shift of hemoglobin from relatively abundant types of red cells (i.e., A) to universally compatible forms. Unfortunately, since the half-life of solutions made from human-derived hemoglobin is short (i.e., hours), the need for red cell transfusion may merely be delayed and not eliminated by its use. Such solutions might be useful in an acute intraoperative hemorrhage, but RBCs would probably still be required in the postoperative period. If this occurred, the patient would be exposed to the risks and cost of both blood substitute administration and RBC transfusion. There would also be loss of some hemoglobin in the harvest, processing, and production steps in this strategy. Therefore, several questions remain about the utility and practicality of this approach.

Just as the first hemoglobin solutions used bovine hemoglobin, so do several solutions currently under development (45,46,67). The oxygen affinity (P_{50}) of bovine hemoglobin is similar to human hemoglobin and is not controlled by 2,3-DPG but instead by chloride ion which is present in large concentrations of the plasma (68–70). When human hemoglobin is removed from red cells, the effect of 2,3-DPG on the oxygen/hemoglobin dissociation is lost. However,

since the oxygen/dissociation curve of bovine hemoglobin is regulated primarily by chloride ions, its dissociation curve can shift normally outside of red cells. There is also a more pronounced Bohr effect in bovine hemoglobin, resulting in enhanced delivery of oxygen to tissues at a lower pH (71). Another attractive feature of bovine hemoglobin is its abundance. A 500-kg steer has approximately 35 L of blood containing approximately 12 g/dL of hemoglobin for an approximate total body hemoglobin content of 4.2 kg. A herd of 20,000 cattle could be routinely phlebotomized to provide the hemoglobin needed for a substantial amount of hemoglobin-containing solutions (64). However, governmental regulatory agencies in several countries are concerned about the possibility of interspecies transmission of infectious disease, such as bovine spongiform encephalitis. The potential for interspecies disease transmission is poorly understood. In this context, it should be noted that hemoglobin can be successfully purified from human RBC units containing the HIV virus (72).

Recombinant DNA technology has been used to produce modified human hemoglobin molecules in *Escherichia coli* and *Sarcomyces cerevisiae* (53–55,73,74). Unfortunately, it is unclear whether the yield of hemoglobin per unit of microorganism is sufficient to make large scale commercial production of hemoglobin possible. There are also concerns about complete separation of bacterial components from the hemoglobin and waste management of the byproducts of its production (55).

Another biotechnologic approach to producing large amounts of hemoglobin involves transgenic manipulation of animals to produce RBCs that contain a substantial proportion of human hemoglobin (75,76). This approach has been attempted in pigs, and approximately 50% human hemoglobin has been produced in these animals. This approach is noteworthy because it could provide an abundant source of human hemoglobin.

Physiologic Effects. When animals are given hemoglobin solutions in exchange transfusions, oxygen consumption and carbon dioxide production do not change, cardiac output remains the same, and increases in mean arterial pressure, pulmonary artery pressure, and systemic vascular resistance are seen (Figure 3) (33,44,45,77–79). Long-term survival (7–30 days) is possible after partial and complete exchange transfusion with hemoglobin solutions (79–82). After complete exchange transfusion, there is regeneration of native red blood cells. In these cases the iron made available by the hemoglobin solution may aid hematopoiesis and speed the return of RBCs.

In animal models of hypovolemic, hypotensive hemorrhagic shock, hemoglobin solutions restore circulating blood volume and provide adequate tissue

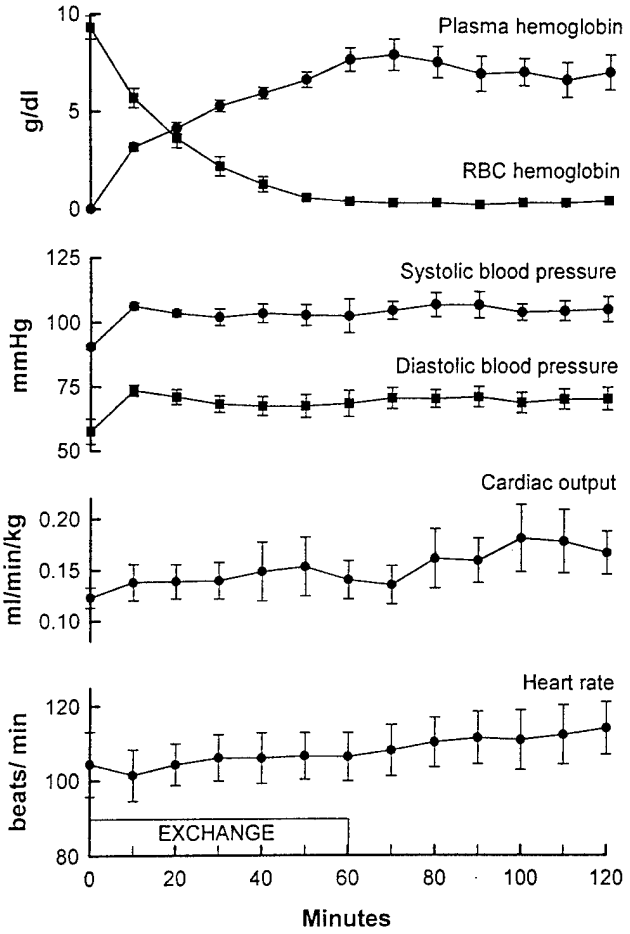

Figure 3. Hemodynamic impact of exchange transfusion of α-α cross-linked hemoglobin in a conscious swine model. In this experiment chronically instrumented swine underwent a total exchange transfusion during which their native blood volume was replaced with a cross-linked hemoglobin solution. The top panel demonstrates the time course of red blood cell (RBC) hemoglobin loss from the circulation along with the increase of free hemoglobin associated with the exchange transfusion. The second panel demonstrates that the exchange transfusion was associated with an increase in both systolic and diastolic blood pressures. This increase was highly significant ($P < 0.05$) and observed in the 28 animals studied. The bottom two panels demonstrate that the hypertension observed during the exchange transfusion was not associated with marked changes in cardiac output or heart rate. [Figure adapted from Hess et al. (84).]

oxygenation (46,83,84). The use of hemoglobin-containing solutions compared to crystalloid or colloid solutions has been associated with improved survival when animals have been subjected to acute blood loss and resuscitated (65,85). Several studies have suggested that oxygen delivery is similar with transfusion of hemoglobin solutions and RBCs (45,78,83,86). However, these experimental models used venous oxygen saturation as an indicator of end-organ oxygenation. Recent studies by Winslow et al. (87) that directly measured tissue oxygen content with indwelling electrodes, suggest that tissue oxygenation

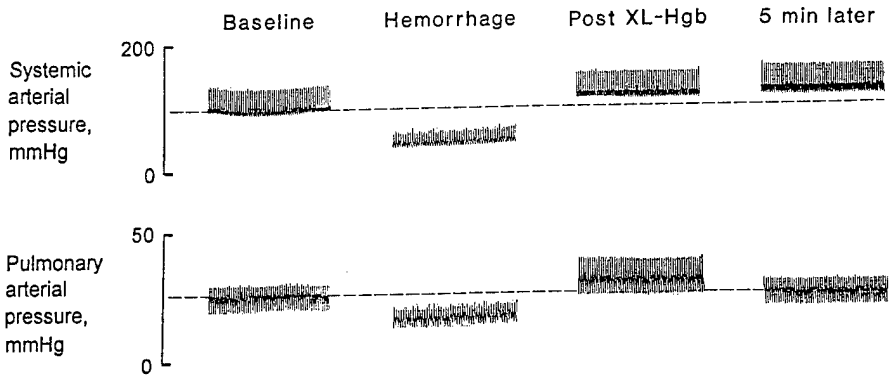

Figure 4. Individual record of a barbiturate-anesthetized dog demonstrating the pressor effects of hemorrhage and transfusion with a cross-linked hemoglobin solution. Prior to baseline measurements the animals received systemic α and β blockade so that the impact of changes in arterial pressure on reflex control in the circulation would be minimized. The dog's blood volume then was reduced by approximately one-third. This magnitude of hemorrhage caused marked reductions in both systemic and pulmonary artery pressures. Immediately after hemorrhage the animal was transfused over 1–2 min with an equal volume of cross-linked hemoglobin solution. This transfusion caused an immediate increase in systemic and pulmonary artery pressures. Five minutes after the replacement transfusion there was a further increase in systemic arterial pressure. Post XL Hgb = post cross-linked hemoglobin. (From Dietz et al. Unpublished observation.)

can be significantly lower after transfusion of hemoglobin solutions compared to RBCs despite similar mixed-venous oxygen saturations. These studies highlight the need for better physiologic indicators of actual oxygen delivery to tissues.

Increases in mean arterial and pulmonary artery pressures and systemic and pulmonary vascular resistances have been reported with the infusion of most hemoglobin solutions (45,83–86,88). Figure 4 demonstrates the time sequence of these changes in an anesthetized dog being transfused with α-α cross-linked hemoglobin. The increases in these pressures appear to be greater than expected from the restoration of normovolemia. This additional pressor effect may be related to the binding of nitric oxide (NO) by the free hemoglobin molecule (89–92). NO is a potent vasodilator that is synthesized in and released by the vascular endothelium and nonadrenergic, noncholinergic vasodilator nerves (93,94). There is continuous release of NO by vascular endothelium, and NO contributes to the maintenance of normal systemic and pulmonary arterial blood pressures (95). Additionally, the hemoglobin molecule itself may possess vasoconstricting properties (96,97).

The mild-to-moderate vasopressor effect associated with transfusion of hemoglobin solutions may be beneficial in the setting of hypovolemic shock and other clinical situations that usually warrant RBC transfusion. An increase in mean arterial pressure, in conjunction with decreased viscosity of hemoglobin solutions, may improve oxygen delivery by hemoglobin solutions in various vascular beds (98). For example, in a rat model of brain ischemia, infarct size is reduced in the presence of a hemoglobin solution (99–101). However, much more needs to be known about how this pressor effect interacts with common coexisting diseases frequently seen in older surgical patients (e.g., renal insufficiency, coronary artery disease, hypertension, etc.) (102) before this "side effect" can be considered beneficial.

In animal models, transfusion with highly purified solutions of modified hemoglobin can be associated with mild transient increases in blood urea nitrogen and creatine (103). Precipitation of trace amounts of free hemoglobin can be found in the kidneys (103). The vasopressor effect seen with transfusion of hemoglobin solutions may affect renal blood flow and its distribution. Although no long-term renal damage has been seen in young, healthy animal models or healthy human volunteers, it is unclear whether these renal effects will also be mild and transient in older patients who have reduced baseline renal function (104).

Unmodified, purified human hemoglobin that is free from RBC cellular components has no apparent immunogenicity (65). Hemoglobin that is modified by nonspecific cross-linking agents can, however, be antigenic under some circumstances (105,106). After repeated exposure of dogs and rabbits to heterologous hemoglobin solutions, in conjunction with maneuvers designed to maximize their immune responses, intravenous administration of heterologous polyhemoglobin or stroma-free hemoglobin solutions resulted in antibody responses significantly greater than in control animals (105). In some forms (e.g., conjugated), heterologous hemoglobin is much less immunogenic (107). Feola et al. (67) reported that single large infusions of modified or unmodified bovine hemoglobin in rabbits produced no detectable antibodies. Other hemoglobins which have been tested for possible antigenicity include those of sheep, rabbits, and dogs (108). These animal hemoglobins were found only

weakly antigenic in the various species. Further studies are required to determine whether either single or repeated infusions of heterologous hemoglobin will evoke a clinically significant immune response in humans (109).

Like saline, hemoglobin and polyhemoglobin solutions have minimal direct effect on coagulation. In an animal model, replacement of 10% of the blood volume with hemoglobin solution (7 g/dL) did not significantly alter prothrombin time, partial thromboplastin time, factor X, fibrinogen, antithrombin III, antiplasmin, or plasminogen when compared to saline control (110). Hemoglobin solutions do not affect adenosine diphosphate-induced platelet aggregation or platelet activation (110,111). Surprisingly, the effects of massive transfusions of hemoglobin solutions and concurrent administration of other blood products on coagulation profiles has not been studied extensively.

Purified hemoglobin solutions have little effect on complement activation. There are no differences in C_3 or C_{3a} levels between plasma incubated with hemoglobin solutions and saline controls (112). However, endotoxin and membrane stroma found in unpurified hemoglobin solutions significantly increase C_3 and lower C_{3a} (112). Although purified stroma-free hemoglobin and polyhemoglobin do not activate complement, contaminants such as membrane stroma or endotoxin do activate the complement system.

Biodistribution. Biodistribution studies of isotope-labeled modified hemoglobin show that modified hemoglobin is scavenged mainly by the reticuloendothelial system (RES) (66,104,113,114). The long-term effects of increased whole body uptake of free hemoglobin have not been established. Due to the efficiency of the RES, intravascular retention time averages 6–8 h with most hemoglobin solutions (43,115). The iron liberated from the breakdown of hemoglobin solutions appears to be recycled and may facilitate hematopoiesis (116).

Status of Current Trials and Applications. A variety of hemoglobin-based, oxygen-carrying volume expanders have been developed by the pharmaceutical industry and several are in Phase I and II clinical trials. Several solutions have been used in healthy anesthetized patients who are undergoing surgical procedures. Companies currently are developing variables for Phase III trials. In addition to the obvious uses for intraoperative blood restoration and volume resuscitation for hemorrhage, proposed uses of hemoglobin solutions include cardioplegia, bypass pump prime during extracorporeal circulation, perfusion of organs awaiting transplantation, perfusion of ischemic microvascular beds, and enhancement of tumor susceptibility to radiotherapy (Table 1). These potential clinical applications will be discussed later.

Table 1. Blood Substitutes: Potential Clinical Applications

Routine perioperative use
Volume resuscitation during trauma
Perioperative hemodilution
Augmented oxygen delivery to ischemic tissue
Treatment of hypotension associated with septic shock
Transfusions in people with multiple antibodies
Increased oxygen delivery to radiosensitive tumors

Liposome-Encapsulated Hemoglobin (LEH)

In 1957 Chang (59) first proposed encapsulation of hemoglobin within a lipid-based pseudomembrane or liposome. He theorized that an encapsulated hemoglobin molecule would have fewer side effects, longer intravascular duration, and greater oxygen-carrying capacity. The hemoglobin tetramer encapsulated within a liposome is more resistant to dissociation into its dimeric form. Several LEH formulations have been developed that can sustain life in experimental animals whose hematocrits have been reduced to levels otherwise incompatible with survival (117–121).

Structure. The typical form of synthetic erythrocyte is an unilamellar liposome containing stroma-free hemoglobin solution as encapsulated fluid (122–126). The membrane, which is the only truly artificial part of the typical synthetic erythrocyte, is composed of a phospholipid bilayer, with molecules of cholesterol added for increased rigidity and mechanical stability (122,124). The addition of 2,3-DPG or inositol hexaphosphate adjusts the oxygen dissociation curve and P_{50} to match that of blood (123).

There are several methods of manufacturing LEH solutions. They differ in the various technologies used for formation of the liposomes and the actual lipids used for incorporation into the membrane. It remains to be seen which manufacturing processes might lead to a clinically useful product. Despite the potential theoretical advantages of LEH solutions, their development is still in the early stages in comparison to the simple hemoglobin solutions and the perfluorocarbons.

Limitations. One continuing problem of LEH solutions is uptake of the liposomes by the RES (117,125). Disruption of the RES after LEH solution administration could lead to reduced resistance to infections. Indirect observations from massive transfusion studies in rats indicate that fatal infections are a problem after LEH solution administration (117). Attempts are now directed at modifying the membrane to increase the plasma half-life of LEH solutions by decreasing uptake by the RES (124). There are currently no major efforts by the

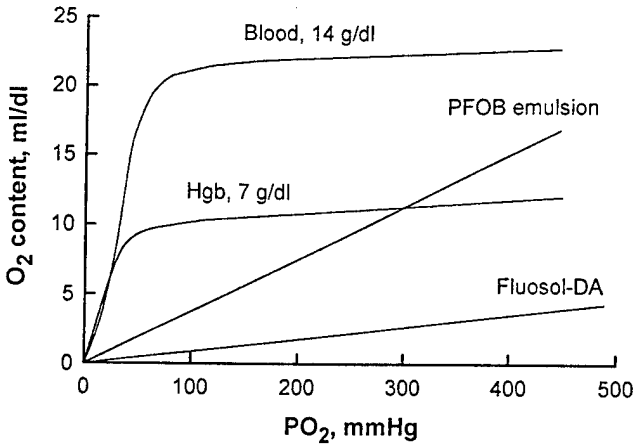

Figure 5. Comparison of the oxygen-carrying capacity of fluorocarbon and hemoglobin-based blood substitutes in comparison to whole human blood. Whole blood with a hemoglobin content of 14 g/dL possesses an arterial O_2 content of 20 mL/dL and a Po_2 of 100 mm Hg. Hemoglobin-based blood substitutes, which have normal hemoglobin O_2 dissociation curves, have an arterial O_2 content of 10 mL/dL when the hemoglobin concentration is 7 g/dL. By contrast, fluorocarbon emulsions require a much higher arterial Po_2 to achieve high levels for arterial O_2 content. A 90% perfluorooctylbromide (PFOB) emulsion can carry 10 mL of O_2/dL at a Po_2 of 300 mm Hg. Perfluorodecalin (Fluosol DA 20™), which used early emulsification technology to achieve a 20% fluorocarbon emulsion, can only carry 2–3 mL of O_2/dL at an arterial O_2 tension of 300 mm Hg. [Figure adapted from Winslow (12).]

pharmaceutical industry to develop LEH solutions, and no clinical trials are underway in humans.

Perfluorocarbons

In 1966, Clark and Gollan (127) demonstrated the oxygen-carrying capacity of fluorocarbon emulsions when they reported the survival of mice immersed in a perfluorochemical solution for extended periods of time (127). Perfluorocarbons are synthetic compounds that act as solvents for oxygen molecules. Typical fluorocarbon compounds can dissolve 40–50 vol% of oxygen at a partial pressure of oxygen of 160 mm Hg and 37°C (128–130). The oxygen content of perfluorocarbons is directly proportional to oxygen partial pressure (129). Although insoluble in water, perfluorocarbons can be infused if emulsified and prepared with surfactants. Figure 5 compares the oxygen-carrying capacity of whole blood and various blood substitutes.

First-Generation Products. Currently 20% perfluorodecalin (Fluosol-DA 20™; Green Cross Corp., Osaka, Japan) is the only oxygen-carrying volume expander approved for use in the United States. This approval is restricted to perfusion of coronary arteries after percutaneous transluminal angioplasty (PTCA) (131), but there are reports of use in patients who refuse blood transfusions on religious grounds (132,133). The utility of this product is limited by its low oxygen-carrying capacity, short intravascular persistence, poor shelf-life, temperature instability, and side effects which include marked uptake by the RES and disruption of normal pulmonary surfactant mechanisms (29,134).

The oxygen-carrying capacity of Fluosol-DA 20™ is limited because only a small amount of this perfluorocarbon can be successfully emulsified into solution. Additionally, the perfluorocarbon particles in solution are large. This size decreases the surface area-to-volume ratio and limits the perfluorocarbon/plasma interface. The perfluorocarbon particles in early perfluorocarbon emulsions also tended to coalesce into even larger particles. Surfactants were developed to prevent coalescence, but these interfered with the function of pulmonary surfactant and resulted in lung hyperinflation (29,135). Attempts are now being made to develop alternative surfactants that do not interfere with the function of the lung or other tissues. The second generation perfluorocarbons have a smaller particle size, making it possible to increase both the concentration of perfluorocarbon in solution and the overall volume of perfluorocarbon/plasma interface (136). These changes result in greater oxygen-carrying capacity and enhanced ability to unload oxygen at the tissues. Smaller particle size has also resulted in prolonged intravascular persistence (130)

Products Under Development. The first generation fluorocarbons use a synthetic polymer (Pluronic F-68; ICI Americas Inc., Wilmington, DE) as a surfactant, are rather dilute (20% emulsified fluorocarbon by weight), and have insufficient stability for storage in a ready-to-use form. Some second generation products contain up to 100% fluorocarbon by weight and much higher oxygen-carrying capacities (Figure 5).

For example, perfluorooctylbromide (PFOB), a second generation perfluorocarbon being rigorously investigated because of its stability and high excretion rate (137), has a much higher oxygen-carrying capacity than Fluosol-DA 20™ (Figure 5). Initial animal experiments have shown that PFOB, used as an adjunct to autologous transfusion, maintains tissue oxygenation and hemodynamics during acute normovolemic hemodilution.

Shelf stability of refrigerated, concentration PFOB emulsions of at least 4 yr has recently been demonstrated. This product also contains surfactants that utilize egg yolk phospholipids. These phospholipids are widely used in parenteral nutrition and appear to interfere less with pulmonary surfactant than did earlier surfactants (130). Like hemoglobin solutions, perfluorocarbon solutions have low viscosities which give them rheologic characteristics that may enhance oxygen delivery to ischemic tissues (128).

Metabolism. Intravascularly administered fluorocarbons are excreted unmetabolized by exhalation and also cleared from the circulation by phagocytosis and subsequent uptake in the RES, from which they are progressively excreted through the lungs (29). Their

rate of excretion via the lungs is an exponential function of their molecular weight. The RES mechanism of excretion results in a temporary increase in the weight of the liver and spleen, and a slight increase in liver enzymes is noted (138,139). Animal studies with PFOB infusions have shown PFOB to prolong the effects of barbiturates and alter metabolism of drugs such as lidocaine and tamoxifen (140–142). Because perfluorocarbons are not metabolized, there are no potential toxins. No cytotoxicity or antigenicity of these emulsions has been reported.

Limitations. Although second-generation fluorocarbons have much greater oxygen-carrying capacities, their dissolved oxygen contents at ambient partial pressures are still limited (Figure 5). These products also have limited shelf-lives unless refrigerated or frozen (143). The cause of the limited half-lives is coalescence of these emulsions into progressively larger droplets and less surface area through which to diffuse oxygen. Manufacturers are trying to reduce the fluorocarbon/water interfacial tension by using various surfactants. These manipulations may improve shelf stability and also prolong intravascular persistence.

Side Effects. Studies done on the first-generation surfactant, Pluronic F-68, suggested that this compound interfered with host defense mechanisms as a result of its uptake by the RES (144,145). Pluronic F-68 causes a species-dependent, anaphylactoid-type reaction related to activation of complement (30). It also decreases blood flow and impairs neutrophil chemotaxis. Platelet aggregation has been reported with Pluronic F-68–containing emulsions. Such reactions have not been observed when egg-yolk phospholipids have been used as surfactants (130).

Preliminary clinical studies with egg-yolk phospholipid-based emulsions have shown some side effects. Acute and transient facial flushing and backaches occur during the infusion period, with a secondary delayed response consisting of fever and chills, headaches, and sometimes nausea. This constellation of responses has been described as "flu-like" (R. K. Spence, University of Medicine and Dentistry of New Jersey, unpublished data, 1994). It is felt that these reactions are part of the natural response of the body to clear emulsion droplets from circulation. There is also monocyte and macrophage activation that involves the production and catabolism of arachidonic acid and subsequent early release of prostaglandins and endoperoxides, with later release of cytokines which are believed to cause a delayed febrile response (146).

Status of Current Trials and Applications. Fluosol-DA 20™ has been approved for use during PTCA for high-risk patients (131). It is also being evaluated as an adjunct to cancer chemotherapy (147–149) and for treatment of myocardial infarction in conjunction with thrombolytic therapy (131). The oxygen-carrying capacity of second-generation perfluorocarbon emulsions such as PFOB has been demonstrated. Further progress with newer perfluorocarbons will depend on the development of new surfactants that allow for longer shelf-lives and increased intravascular persistence. The side effects (i.e., flu-like symptoms) associated with infusion of PFOB will need to be addressed prior to widespread use in humans.

Clinical Applications

A variety of potential clinical applications have been proposed for blood substitutes. These are summarized in Table 1. Potentially important applications include routine perioperative use, trauma, intraoperative hemodilution, perfusion of ischemic organs, septic shock, cardioplegia, and tumor therapy.

Routine Perioperative Use

Since most RBCs are transfused in the perioperative period, there are many routine surgical procedures during which an anesthesiologist may expect to transfuse 1–3 U of RBCs to replace normal surgical blood loss (150–153). These transfusions often are guided by hemoglobin or hematocrit values, the clinical status of the patient, and the anesthesiologist's experience and expectations with the specific surgeon and type of procedure (154). In general, these transfusions are undertaken in an effort to maintain adequate oxygen-carrying capacity and are given prior to any significant deterioration of the patient's clinical status (154,155). Efforts to define criteria for transfusion under all circumstances may be impossible. In many situations, blood substitutes might be transfused instead of RBCs.

A major problem with perioperative use is that intravascular persistence of currently available blood substitutes is short compared to that of transfused RBCs. In most perioperative situations, it is likely that the oxygen-carrying capacity provided by a blood substitute would be lost from the circulation in 1 day or less. By contrast, regeneration of native oxygen-carrying capacity via hematopoiesis often takes weeks or more, and the need for RBCs would not be eliminated. If blood substitutes were used intraoperatively, the location of RBC transfusions might be shifted out of operating rooms where patients are closely monitored. Such shifting might potentially expose unmonitored patients to dangerous levels of anemia and also the costs and risks of both blood substitutes and subsequent RBC transfusions. As product development continues, and if efforts to increase the intravascular persistence of these compounds are successful, blood substitutes could replace limited RBC transfusions for routine surgical use.

Trauma

The objective of fluid therapy for hypovolemic, hemorrhagic shock is to reestablish systemic oxygen delivery. This objective is accomplished by volume expansion to restore cardiac output and administration of RBCs to maintain arterial oxygen-carrying capacity. In this context, early treatment of shock trauma with a hemoglobin solution may allow some individuals to be stabilized without receiving RBCs. This might result in a net saving of RBCs in some trauma patients. Many hemoglobin-based solutions have been shown to be effective in the resuscitation of animals in hemorrhagic shock (46,82–85). Most studies use animal models with hemorrhagic loss of approximately 30%–40% of their blood volume. In these models, volume restoration is much more important than replacement of oxygen-carrying capacity to improve outcomes. Indeed, the need for RBC replacement to treat this degree of blood loss is still controversial (156). To show that blood substitutes are superior to conventional plasma expanders (e.g., lactated Ringer's solution, saline, hetastarch, human serum albumin, etc.) and equivalent to RBC replacement, a more severe model of hypovolemic hemorrhagic shock has been used (80). Initial studies which restored a two-thirds blood volume hemorrhage with modified hemoglobins showed enhanced survival compared to standard volume expanders or recently emerging hypertonic/hyperoncotic solutions. The safety of long-term massive transfusion with hemoglobin solutions in humans is not yet known. At this time it is unclear whether perfluorocarbon emulsions are appropriate for use in massive transfusion.

Perioperative Hemodilution

One potentially useful blood conservation strategy is hemodilution (157). In this technique, several units of whole blood are removed from the patient at the beginning of surgery. Blood volume is replaced with intravenous fluids and the blood reinfused as needed during surgery. It is unclear whether this technique is appropriate or effective in reducing the use of banked blood (153,158,159). It is also potentially expensive and the cost/benefit ratio may be high (31,32). The hope is that replacement of the blood collected at the start of surgery with a blood substitute would allow collection of more blood and increase the efficacy of hemodilution, thus limiting the use of banked blood. This approach circumvents the problems associated with the short plasma half-lives of many of the products under development. In addition, the outcome of some surgical procedures might be improved if blood viscosity can be reduced and flow through small vessels increased. Blood substitutes might also be effective as primes for the cardiopulmonary bypass circuit during cardiac surgery (160). There is also consideration being given to using hemoglobin solutions in preoperative blood donation practices to increase the total amount of blood collected (161).

Perfusion of Ischemic Tissue

With their small particle size and low viscosity, hemoglobin or perfluorocarbon red cell substitutes may bypass circulatory obstructions or utilize collateral microcirculation to penetrate and reoxygenate hypoxic tissue beds. This possibility has been demonstrated in animal models of cerebral ischemia (99–101). Other examples might include treatment of ischemic ulcers in diabetics, treatment of ischemic crises in sickle-cell anemia patients, or perfusion of devascularized tissues prior to revascularization. A study performed in sickle-cell patients who suffered vaso-occlusive crises demonstrated reversal of the ischemic crisis and stimulation of the hematopoietic system when these patients were treated with hemoglobin-based blood substitutes (116). There are also reports demonstrating improved healing of ischemic intestine when bathed in oxygenated perfluorodecalin (162–164). In animal studies, amputated limbs perfused with fluorocarbon emulsions displayed less ischemic insult than those preserved in traditional solutions (165,166).

The fluorocarbon emulsion Fluosol-DA 20™ has been evaluated and approved by the United States Food and Drug Administration for intraarterial use during PTCA to protect myocardial tissues while the balloon is inflated (131), and the second generation PFOB is being studied in the same clinical setting (167). Solutions of modified hemoglobin have also been tested during coronary angioplasty (168). In addition to use during PTCA, preclinical data indicate enhanced thrombolytic activity when Fluosol-DA 20™ is used in conjunction with intracoronary tissue plasminogen activator (169). Neither of these applications of Fluosol-DA 20™ is widely used clinically.

Septic Shock

It has been hypothesized that NO plays a major role in hypotension after exposure to endotoxin (170–172). Once induced by cytokines, NO synthase continues to produce NO even after removal of the stimulus (173–175). Approaches to the treatment of septic shock are being developed which prevent endotoxin from activating leukocytes that start the inflammatory response (176–178). Unfortunately, blockade of the cytokine receptor interaction does not have an immediate effect on NO production by NO synthase. There have been some promising animal studies and human case reports showing that administration of arginine analogs to block NO synthase can dramatically reverse the hypotension and vasodilation seen with septic shock

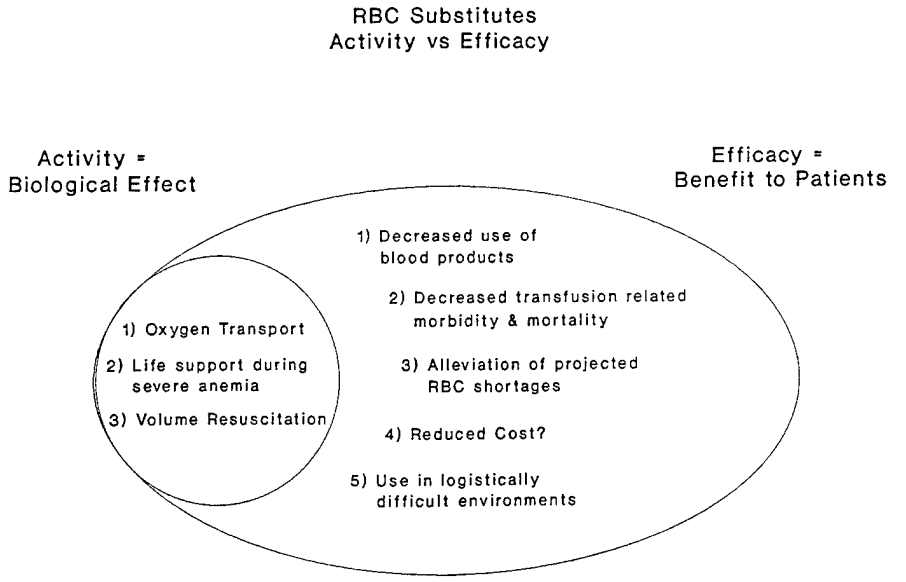

Figure 6. This schematic outlines concepts related to blood substitute activity versus efficacy. Activity is the demonstration of a biologic effect. In the case of blood substitutes, key biologic effects include oxygen transport, life support during severe anemia, and adequacy during volume resuscitation in models of hypotensive hemorrhage shock. These effects are listed in the left circle. By contrast, efficacy is demonstration of benefit to patients. Benefit to patients would include decreased use of blood products, decreased transfusion related complications, and potentially attractive logistic considerations associated with blood substitutes. All compounds that ultimately show efficacy also need to possess adequate biologic activity. However, all compounds which demonstrate biologic activity may not prove to be effective blood substitutes. RBC = red blood cell.

(179–184). Administration of hemoglobin solutions which avidly bind NO may be administered in pharmaceutically effective amounts to remove NO and prevent or treat hypotension in septic shock (185,186).

Patients with Multiple Antibodies

There are a few patients with multiple antibodies to red cell surface antigens. These antibodies are usually present because of multiple previous transfusion exposures in patients needing ongoing transfusions. In such patients, not only is compatible blood difficult to find, but transfusion of RBCs poses serious risk for fatal transfusion reactions. For such patients, blood substitutes could potentially replace RBCs in a transfusion regimen similar to the hemodilution protocol previously described. However, the short intravascular persistence of available products severely limits their utility as an actual long-term replacement of RBCs.

Tumor Therapy

Hypoxic cells exist in solid tumors, and these cells are relatively resistant to the cytotoxic effects of ionizing radiation (187–189). Thus, the hypoxic cell population limits the curability of experimental animal tumors that are subjected to large doses of ionizing radiation (190). Since hypoxic cells may be either noncycling or slowly progressing through the cell cycle (191–193), they are also presumed to be relatively resistant to cell cycle-specific chemotherapy. It is hypothesized that tumor therapy could capitalize on synthetic oxygen-carriers' property of low viscosity. This ability to better perfuse tumor microvasculature would enhance tumor oxygenation, thus increasing susceptibility to both ionizing radiation and chemotherapy (194–196).

Tumor growth in mice has been blunted by radiation given after infusion of perfluorochemical emulsions (197–199). In humans, Fluosol-DA 20™ has been used in conjunction with 100% oxygen as an adjuvant to radiation therapy in advanced squamous cell tumors of the head and neck (147), advanced non-small cell carcinoma of the lung (148) and primary high-grade brain tumors (149). Although the Fluosol-DA 20™ treatment regimen has been well tolerated, no improvement in length or quality of survival has been demonstrated.

Efficacy

Efficacy is the demonstration of clinical benefit to patients and is frequently confused with the concept or demonstration of biologic activity (200,201). Many products now under development are biologically active. They can transport oxygen and sustain life in the absence of RBCs, but will they be able to be used clinically in a way that reduces the use of banked blood? Figure 6 demonstrates the relationship between activity and efficacy. Although there may be some limited novel uses of oxygen-transporting solutions for some unusual disease states or experimental therapies, a solution that could "replace" red cells is the goal of most products now being developed. If a blood substitute can be manufactured and administered in a cost-effective manner, if such a product has a side effect profile equal to or better than banked blood, and if this product can reduce the use of banked blood, it will clearly possess great efficacy as a routine substitute for RBC transfusion.

Rigorous criteria for efficacy may be a difficult hurdle for many products now under development to

meet. The existence of side effects, such as vasoconstriction and hypertension, may limit their use, especially in elderly patients who frequently have various coexisting diseases. The short plasma half-lives of these products provide short-term oxygen-carrying capacity but may merely delay the transfusion of RBCs. Large clinical trials will have to be conducted to demonstrate reduced use of red cells when blood substitutes are administered.

Summary

Oxygen-carrying volume-expanding solutions that can sustain life in the absence of red blood cells have been developed. Concerns about side effects, sources of hemoglobin, and the ultimate demonstration of efficacy will have to be satisfactorily addressed before anesthesiologists routinely administer such solutions in place of red cells during surgery.

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EFFECTS OF CROSS-LINKED HEMOGLOBIN ON REGIONAL VASCULAR CONDUCTANCE IN DOGS

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ABSTRACT

Hemoglobin-based blood substitutes cause systemic vasoconstriction. To determine if an α-α cross-linked hemoglobin solution (XL-Hgb) interferes with nitric oxide (NO)-mediated vasodilation, we studied vasodilator responses to acetylcholine (ACh) and sodium nitroprusside (NTP) in the femoral, superior mesenteric, and circumflex coronary arteries before and after partial exchange transfusion with XL-Hgb in anesthetized dogs (N=6). These responses were compared to treatment with 5% albumin (N=6). Prior to XL-Hgb administration MAP was 81±5 and increased to 112±8 mmHg after transfusion. MAP was 84±4 mmHg before and decreased to 76±4 mmHg after albumin (both $p<0.05$ vs baseline MAP). Vascular conductance after XL-Hgb decreased in the femoral artery (1.48±0.21 to 0.68±0.09 $ml \cdot mmHg^{-1} \cdot min^{-1}$; $p<0.05$), was not changed in the mesenteric bed, and increased in the coronary artery (0.19±0.03 to 0.26±0.02 $ml \cdot mmHg^{-1} \cdot min^{-1}$; $p<0.05$). After albumin, conductance was unchanged in the femoral artery, but increased in both the mesenteric (3.30±0.30 to 5.00±0.45 $ml \cdot mmHg^{-1} \cdot min^{-1}$) and coronary (0.25±0.02 to 0.49±0.03 $ml \cdot mmHg^{-1} \cdot min^{-1}$) beds (both $p<0.05$). The progressive vasodilation observed with increasing doses of ACh in the femoral artery was unaffected by either treatment. In the mesenteric bed, XL-Hgb had no effect on the responses to Ach, but these were augmented after albumin. In the coronary bed XL-Hgb blunted and albumin augmented the dilator responses to Ach. The vasodilator responses to NTP were also blunted in the coronary circulation after XL-Hgb. In five additional dogs the NO synthase inhibitor N^G-monomethyl L-arginine (L-NMMA) caused baseline mean arterial pressure to rise from 85±4 to 90±8 mmHg ($p<0.05$), and blunted the coronary dilator responses to ACh. Subsequent XL-Hgb administration caused a further rise in mean arterial pressure to 112±19 mmHg ($p<0.05$) and also

further blunted vasodilator responses in the coronary circulation. XL-Hgb appears to have highly complex effects on regional and systemic circulation; however, it clearly blunts the vasodilator response to ACh and NTP in canine coronary arteries *in vivo*. The continued responsiveness of the femoral and mesenteric beds to ACh and NTP indicates that these beds retain the ability to dilate to nitric oxide in the presence of XL-Hgb.

Key words: blood substitute

hemoglobin

nitric oxide

vasodilation

coronary arteries

INTRODUCTION

Hemoglobin-based solutions have been developed for use as possible "blood substitutes" (27). The concept is that such solutions could be used in place of packed red blood cells in clinical situations that warrant both volume resuscitation and increased oxygen-carrying capacity (27). These products are also attractive because they could limit the transfusion-related transmission of infectious diseases, and potentially address a variety of logistical issues related to the collection, storage, and administration of red blood cells (27,28). These issues along with age-related demographic pressure on the blood supply (24) also make the use of a safe and effective "blood substitute" attractive in patients undergoing a variety of elective surgical procedures.

A number of studies have shown that hemoglobin-based solutions can sustain life in animal models in the absence of red blood cells (2,10,12,20). There have also been demonstrations of improved outcomes in animal models of hypovolemic, hypotensive shock (5,19). However, one commonly noted physiologic effect usually observed after administration of these compounds is moderate (10-30 mmHg) arterial hypertension (3,9,10,12,16,19,25,26). This hypertension, caused by generalized vasoconstriction, is thought to be secondary to free hemoglobin molecules scavenging the vasodilating substance nitric oxide (NO) (1,14). However, little information is available on how hemoglobin solutions affect vasodilation caused by pharmacologic stimulation of NO release or via exogenous administration of nitrovasodilator compounds.

With this information as a background, we studied the effects of both volume resuscitation and volume loading with α-α cross-linked hemoglobin (XL-Hgb) on femoral,

superior mesenteric, and circumflex coronary vascular conductance in an anesthetized canine preparation. We also studied the vasodilator responses in these vascular beds to intraarterial administration of acetylcholine (ACh) and sodium nitroprusside (NTP) in an effort to determine how XL-Hgb affects the *in vivo* responsiveness of various vascular beds to endogenous and exogenous NO. We hypothesized that if the primary vasoconstrictor effects of XL-Hgb are due to NO scavenging, then XL-Hgb administration would blunt the vasodilator responses to both ACh and NTP.

MATERIALS AND METHODS

Animals and instrumentation

Anesthesia. After Institutional Animal Care and Use Committee approval, 21 mongrel dogs of either sex (15-20 kg) were anesthetized with pentobarbital (40 $mg \cdot kg^{-1}$ intravenously followed by an infusion at 5 $mg \cdot kg^{-1} \cdot hr^{-1}$). The dogs were endotracheally intubated and mechanically ventilated ($FiO_2 = 0.3$, $V_t = 20\ ml \cdot kg^{-1}$, RR = 15, PEEP = 5 cm H_2O). Arterial blood gases were monitored at regular intervals and pH was maintained by either adjusting the ventilatory rate (respiratory acidosis) or by addition of intravenous sodium bicarbonate (metabolic acidosis). Care was taken to maintain P_{CO2} between 35-40 mmHg and arterial pH between 7.35 and 7.45 in an effort to minimize the impact of changes in pH on vascular tone. The dogs were placed on a heating pad in the supine position and warming lights were adjusted to maintain core temperature between 36.2 - 39.2°C. The right internal jugular vein was cannulated with an 8 F introducer, and a 7.5 F pulmonary artery catheter was advanced into the pulmonary artery and was used to measure central venous pressure, pulmonary artery pressure, pulmonary capillary wedge pressure, right atrial temperature, and cardiac output. A 15 gauge internal diameter polyethylene tube was placed in the left femoral artery and secured with suture. This catheter was used for measurement of arterial pressure and as a site for withdrawal of blood samples. It was connected to a pressure transducer flushed continuously (3 $ml \cdot hr^{-1}$) with saline containing heparin (2 $U \cdot ml^{-1}$).

Vascular instrumentation. After a right groin incision and exposure of the right femoral artery, an ultrasonic flow probe (Transonic #3R) was placed on the right femoral artery. A 22 gauge, 25 mm Teflon catheter was placed in the right femoral artery just downstream from the flow probe and pointing upstream. A left flank incision was then performed and the superior

mesenteric artery carefully exposed. An ultrasonic flow probe (Transonic #3R or #4R) was placed on the superior mesenteric artery and the artery was cannulated with a 22 gauge, 25 mm Teflon catheter in a fashion similar to the femoral artery. After instrumentation of the femoral and superior mesenteric arteries was complete, the dogs received systemic α-blockade consisting of phentolamine [2 $mg \cdot kg^{-1}$ intravenously followed by an infusion of 1 $mg \cdot kg^{-1} \cdot hr^{-1}$ (4)] and systemic β-blockade with propranolol [2 $mg \cdot kg^{-1}$ intravenously followed by an infusion of 1 $mg \cdot kg^{-1} \cdot hr^{-1}$ (4)]. The purpose of the sympathetic blockade was to insure that any baroreceptor-mediated changes in catecholamine release associated with maneuvers that alter arterial pressure during the experimental protocols did not affect the regional vascular conductance measurements during the interventions. Additionally, β-blockade also allowed heart rate to remain constant throughout the experiment and the slower heart rate facilitated instrumentation of the circumflex coronary artery.

A left thoracotomy incision was then performed at the fifth intercostal space. The pericardium was then opened and the left circumflex coronary artery identified. With meticulous dissection, this vessel was freed from connective tissue and a flow probe (Transonic #2S) was placed around it. A 24 gauge, 19 mm Teflon catheter was placed in the circumflex coronary artery as in the femoral and mesenteric arteries. The catheters in each of the three instrumented arteries were connected to continous saline flush (3 $ml \cdot hr^{-1}$) and were used for the infusion of study drugs. The three ultrasonic flow probes were connected to a digital integrater (Transonic model #206, small animal blood flow meter) which continously displayed blood flow in $ml \cdot min^{-1}$.

Drugs

Cross-linked hemoglobin solution (XL-Hgb) was obtained from the U.S. Army. This solution contains approximately 7 gm·deciliter^{-1} of hemoglobin in the form of a stabilized tetramer suspended in Ringer's acetate solution (11). **Five percent human serum albumin** (albumin; Miles Laboratories,Incorporated, Elkhart, IN) was administered intravenously in some dogs to replace an equal volume of blood. **Phentolamine** (Sigma Chemical Company, St. Louis, MO) was administered intravenously to systemically block the α-adrenergic system (4), and **propranolol** (Ayerst Laboratories Incorporated, New York, NY) to block the β-adrenergic sympathetic receptors (4).

Acetylcholine (ACh; Sigma Chemical Company, St. Louis, MO) was given intraarterially to stimulate muscarinic receptors on the vascular endothelium to release nitric oxide and cause vasodilation (21,23). The femoral, superior mesenteric and circumflex coronary arteries sequentially received ACh in doses of 0.1, 1.0, 10.0 and 100.0 μg·min^{-1}. **Sodium nitroprusside** (NTP; Sigma Chemical Company, St. Louis, MO), a nitric oxide donor, was administered intraarterially to cause non-endothelial dependent vasodilation (13). NTP was given sequentially in the femoral, mesenteric and coronary arteries in doses of 0.1, 1.0, 10.0 and 50.0 μg·min^{-1}. Each dose of each of the drugs was given for 2 min followed by 5 min of rest before a subsequent dose of either ACh or NTP was given. This allowed a return to baseline conditions between doses. The ACh and NTP doses were chosen on the basis of pilot experiments (N=3) which indicated that they provided adequate dose-response relationships. Stability and reproducibility of dose-response relationships were also established in the pilot

experiments. Administration of ACh or NTP was discontinued in any instance where marked hypotension or dysrhythmias ensued.

Sodium meclofenamate (Calbiochem, La Jolla, CA) was used in Protocol 2 to block prostaglandin synthesis and resulting vascular responses. **N^G-monomethyl L-arginine** (L-NMMA; Cal Biochem, La Jolla, California), an arginine analog, was administered in Protocol 2 to inhibit NO-synthase.

Experimental protocols

Protocol 1 (See Figure 1) The purpose of this protocol was to determine if XL-Hgb interferes with NO-mediated vasodilation. A total of 12 dogs were studied. Each dog was instrumented as described above. After instrumentation, dose-response curves for ACh and NTP were obtained for the femoral, superior mesenteric and circumflex coronary arteries. The dogs were then divided into one of two treatment groups. Group 1 dogs (n = 6) had 1/3 of their estimated blood volume (body mass in kg x 85 $ml \cdot kg^{-1}$ x 0.33; Muir 1995)withdrawn over 3 - 5 min via the femoral artery catheter, and this volume was replaced with an equal volume of albumin administered over 3-5 min via the right internal jugular venous introducer. In Group 2 (n = 6), dogs had 1/3 of the blood volume withdrawn and replaced with an equal volume of XL-Hgb, administered over the same time course as the albumin given to the dogs in Group 1. In both groups a 20 min equilibration period followed the blood withdrawal and resuscitation. ACh and NTP dose-response curves were then repeated. Cardiac output and arterial blood gases and hematocrit were measured at baseline, after sympathetic blockade, and prior to each dose-response curve determination, and pH was adjusted as previously described. With each blood

gas measurement, whole blood samples were centrifuged and the supernatant was analyzed to determine plasma hemoglobin content.

Animals were euthanized at the end of each experiment without emerging from anesthesia by intravenous injection of pentobarbital 100 $mg \cdot kg^{-1}$ followed by intracoronary injection of potassium choloride, 200 mEq.

Protocol 2 (See Figure 2) This protocol was designed after completion of Protocol 1 and served to test the hypothesis that the vasoconstriction observed could be accounted for primarily by vascular endothelial NO inactivation due to circulating free hemoglobin. Protocol 2 was conducted in 6 additional animals. After instrumentation as previously described, the dogs received systemic α- and β-blockade, and intravenous sodium meclofenamate 5 $mg \cdot kg^{-1}$ to block possible prostaglandin-induced changes in vasomotor tone. They then underwent determination of ACh and NTP dose-response curves. This was followed by intravenous administration of L-NMMA (5 $mg \cdot kg^{-1}$), and a second determination of the ACh and NTP dose-response curves. Each dog then received an infusion of XL-Hgb equal to 10% of the calculated intravascular volume. After the volume load, a third series of ACh and NTP dose-response curves was determined. Hemodynamic data, blood gas and hemoglobin concentration measurements were collected as described in Protocol 1.

Animals were euthanized at the end of each experiment without emerging from anesthesia by intavenous injection of pentobarbital 100 $mg \cdot kg^{-1}$ followed by intracoronary injection of potassium choloride, 200 mEq.

Statistical analysis

The changes in vascular reactivity before and after the various interventions are expressed as changes in vascular conductance above baseline and are expressed with the units $ml \cdot mmHg^{-1} \cdot min^{-1}$. This approach is necessary since the various experimental interventions caused marked changes in the mean arterial pressure. Conductance was used because it corrects for differences in arterial pressure and is linearly related to changes in blood flow. Data are expressed as mean ± standard error. When dose-response curves are compared, a two-way analysis of variance for treatment and dose was used. For comparison of other variables or single responses before and after a treatment, paired *t*-tests were used.

RESULTS

Protocol 1

Baseline physiologic variables for the two groups of animals are shown in Table 1. Baseline values were obtained after instrumentation and combined α- and β-blockade. Values obtained after transfusion were those after the equilibration period that followed the experimental hemorrhage and volume resuscitation with either albumin or XL-Hgb. Temperature, pH and heart rate did not change in response to the interventions in either group of animals. Mean arterial pressure decreased from 84±4 mmHg to 76±4 mmHg after volume resuscitation with albumin ($p<0.05$). Mean arterial pressure averaged 81±5 mmHg prior to XL-Hgb administration, and increased to 112±8 mmHg when XL-Hgb was administered ($P < 0.05$ vs albumin). Cardiac output was higher in Group 1 dogs after exchange transfusion with albumin (4.3±0.6 vs 2.8±0.3 $L \cdot min^{-1}$; $p<0.05$), but did not change after exchange transfusion with XL-Hgb in Group 2 dogs (Table 1). Hemoglobin was reduced significantly as a result of the experimental hemorrhage. Plasma hemoglobin increased as a result of transfusion with the XL-Hgb.

Figure 3 displays baseline vascular conductance in each of the three arterial beds before and after volume resuscitation. After volume resuscitation **with albumin**, baseline vascular conductance did not change in the femoral artery (1.61±0.33 vs 1.80±0.31 $ml \cdot mmHg^{-1} \cdot min^{-1}$), but increased in both the mesenteric (3.30±0.30 to 5.00±0.46 $ml \cdot mmHg^{-1} \cdot min^{-1}$) and coronary (0.25±0.02 to 0.49±0.03 $ml \cdot mmHg^{-1} \cdot min^{-1}$) vascular beds (both $p<0.05$). After **XL-Hgb infusion**, baseline vascular conductance decreased from 1.48±0.21 to 0.68±0.09

$ml \cdot mmHg^{-1} \cdot min^{-1}$ in the femoral artery ($p<0.05$), was unchanged in the mesenteric artery (2.55 ± 0.37 vs 2.42 ± 0.43 $ml \cdot mmHg^{-1} \cdot min^{-1}$), and increased in the circumflex coronary artery from 0.19 ± 0.03 to 0.26 ± 0.02 $ml \cdot mmHg^{-1} \cdot min^{-1}$ ($p<0.05$) (Fig 1).

The change in conductance from baseline in response to various doses of ACh (dose-response curve to ACh) is summarized graphically in Figure 4 for both the albumin- and the XL-Hgb-transfused dogs. There was no difference in the dose-response curves to ACh in the femoral artery of either albumin or XL-Hgb condition. In the mesenteric and coronary vascular beds after infusion of albumin, there was a significant increase in the conductance response to higher doses of ACh seen when compared to baseline. With XL-Hgb, there was no change in the dose-response relationships in the mesenteric vascular bed; however,there was a significant *decrease* in the ACh-induced vasodilation in the coronary circulation.

Similar changes were observed with dose-response relationships to administration of NTP (Fig 5). There were no significant changes in dose-response relationships in the femoral artery after partial exchange transfusion with either albumin or XL-Hgb. As seen with Ach dose-response relationships, there was increased conductance in the mesenteric vascular bed after administration of albumin, but no change after administration of XL-Hgb. When changes in conductance in the circumflex coronary circulation are examined, there is an increase in conductance after albumin at the lower ($p<0.05$) doses, and a *decrease* in conductance after XL-Hgb is seen over most of the dose-response curve.

Protocol 2

In this protocol, a total of 6 dogs were studied. Data from only 5 animals are included since one dog became hemodynamically unstable during infusion of XL-Hgb and was unable to be resuscitated. Baseline physiologic variables for the three different conditions are shown in

Table 2. As in Protocol 1, baseline values were obtained after systemic α-, β-, and also prostaglandin-blockade. L-NMMA infusion caused baseline mean arterial pressure to rise from 85±4 to 90±8 mmHg ($p<0.05$). With L-NMMA infusion, femoral conductance (0.80±0.20 vs 0.51±0.03 $ml \cdot mmHg^{-1} \cdot min^{-1}$) and superior mesenteric conductance did not change (2.79±0.47 vs 2.78±0.62 $ml \cdot mmHg^{-1} \cdot min^{-1}$) , and circumflex coronary conductance increased from 0.15±0.02 to 0.19±0.01 $ml \cdot mmHg^{-1} \cdot min^{-1}$ ($p<0.05$). Despite varying changes in baseline conductance, there was little impact of L-NMMA treatment on the rise in conductance associated with ACh administration in the femoral or superior mesenteric vascular beds. However, L-NMMA did blunt the vasodilator responses to ACh in the circumflex coronary vessels (Fig 6).

When volume expansion with cross-linked hemoglobin was superimposed on L-NMMA administration there was a further increase in baseline mean arterial pressure to 118±13 mmHg ($p<0.05$). This was associated with decreases in baseline vascular conductance in the femoral (0.51±0.03 to 0.33±0.05 $ml \cdot mmHg^{-1} \cdot min^{-1}$) and mesenteric (2.78±0.62 to 1.89±0.37 $ml \cdot mmHg^{-1} \cdot min^{-1}$) vascular beds (both $p<0.05$), with no baseline change seen in the circumflex coronary circulation (0.19±0.01 to 0.17±0.01 $ml \cdot mmHg^{-1} \cdot min^{-1}$). XL-Hgb did not affect the vasodilator responses to ACh in the femoral or mesenteric vascular beds, but *further* blunted vasodilator responses in the coronary circulation (Fig 6).

DISCUSSION

The principal new finding of this study is that the vasodilator responses to ACh and NTP were blunted in the coronary circulation after volume resuscitation with XL-Hgb but not albumin. Additionally, these altered vasodilator responses appear to be of greater magnitude than seen during blockade of NO synthesis alone. By contrast femoral and mesenteric responses to ACh or NTP were not blunted by administration of cross-linked hemoglobin in spite of the observation that this compound caused hypertension via generalized vasoconstriction with marked decreases in baseline vascular conductance. The physiologic implications and technical limitations of these findings will be discussed.

Protocol 1

Systemic hemodynamic responses

Partial exchange transfusion with XL-Hgb increased mean arterial pressure. Since no change in cardiac output was observed, systemic vasoconstriction accounted for this increase. Exchange transfusion with albumin did not increase MAP, but cardiac output did increase indicating systemic vasodilation. Other studies have reported similar findings of hypertension due to vasoconstriction after transfusion with XL-Hgb (9,10,12,16,19,25,26); however, the magnitude of change is greater in this study compared to others. This may be explained by the systemic alpha and beta blockade which might have limited baroreceptor-mediated vascular and cardiac compensatory responses to the hypertension.

Increased cardiac output was observed after partial exchange transfusion with albumin. This expected increase was probably due both to decreased viscosity allowing for rheologically-enhanced flow conditions and also to decreased oxygen content of blood, which might be associated with metabolic vasodilation in tissue beds in order to maintain delivery of oxygen to

the tissues (22). The viscosity of XL-Hgb solutions is also low, so the viscosity changes were probably similar to those with albumin, but cardiac output did not increase (11). These findings suggest that: 1) the vasoconstricting properties of XL-Hgb have a greater effect on vasculature than viscosity changes, and/or, 2) the oxygen-carrying capacity of the blood was sufficiently maintained with XL-Hgb to negate the autoregulatory vasodilation normally associated with hemodilution and decreased oxygen delivery.

Effects of volume resuscitation with albumin or XL-Hgb on coronary conductance.

After exchange transfusion with albumin there was a 96% increase in baseline conductance in the circumflex coronary artery. This was probably due to the combined effects of decreased viscosity and autoregulatory vasodilation due to decreased oxygen delivery to cardiac tissue. After transfusion with XL-Hgb there was a 37% increase in coronary conductance in spite of evidence for systemic vasoconstriction. This may have been due to complex interactions between decreased viscosity and metabolic autoregulation. Although oxygen delivery to cardiac tissue would be enhanced after XL-Hgb transfusion compared to albumin, there was probably also an increased oxygen demand on the cardiac tissue due to the increased mean arterial pressure (i.e., increased afterload). Thus, viscosity and oxygen delivery mediated autoregulation along with increased metabolic demand probably offset any vasoconstricting properties of the XL-Hgb and contributed to the modest increase in coronary conductance seen after XL-Hgb transfusion.

When dose-response relationships to ACh and NTP in the coronary circulation were compared before and after albumin administration, there was an increase in the responses to ACh at higher ACh doses and an increase in the responses to NTP at lower doses. Overall, however,

the changes were variable. Both the ACh and NTP dose-response curves in the circumflex coronary artery (change from baseline) were similarly blunted after XL-Hgb administration. This might indicate that the XL-Hgb-induced vasoconstriction was due, at least in part, to some action on vascular smooth muscle rather than an effect which was confined to the vascular endothelium. If the endothelium alone had been affected, one would have expected to see changes in the dose-response curve to acetylcholine, since NO release after stimulation of muscarinic receptors is endothelium dependant; but no changes would be expected in the dose-response curve to NTP, which causes non-endothelial-dependent vasodilation. Another possible explanation for the blunting of both the ACh and NTP dose-response curves is that the XL-Hgb acted equally upon all NO present near the vessel wall. In other words, NO, whether released by stimulation of the vascular endothelium or exogenously administered, was rapidly bound by the circulating free hemoglobin molecules.

Effects of volume resuscitation with albumin or XL-Hgb on femoral and mesenteric circulations

Following exchange transfusion with albumin, there was no change in the baseline conductance in the femoral circulation, but there was a 52% increase in the baseline conductance in the mesenteric circulation. This increase along with the augmented increases in conductance seen in the ACh and NTP dose-response curves (Fig 3 and 4) probably reflected the reduced viscosity of the blood. The effects of reduced viscosity on vascular conductance should be magnified at the higher drug doses since viscosity is velocity dependent. As vascular diameter increases the rise in conductance should increase to a greater degree due to the reduced viscosity.

Baseline vascular conductance in the femoral circulation was reduced after XL-Hgb administration, and there was no change in mesenteric conductance. There was also no change in

the dose-response curves to ACh or NTP after XL-Hgb compared to pre-transfusion. This was probably due to a balance of several factors which independently might cause changes in vascular conductance: changes in viscosity leading to enhanced flow, some vasoconstricting effect of XL-Hgb, and some altered level of oxygen delivery sensed by the tissue beds. The observation that XL-Hgb did not alter the vasodilator resonses to ACh and NTP *in vivo* is at odds with previous studies in isolated blood vessels (7). One possible explanation for this is that vasodilator resonses *in vivo* primarily reflect changes in resistance vessel tone while the *in vitro* studies were conducted in rings of conduit arteries. The effects of XL-Hgb on small vessels may differ from the effects on large arteries.

Protocol 2

L-NMMA administration

Administration of sodium meclofenamate, which was used to block any prostaglandin-induced changes in vasomotor tone, did not alter baseline hemodynamic values, baseline vascular conductance or dose-response relationships to ACh in any of the three vascular beds studied.

Administration of L-NMMA caused a small (6%) but significant increase in MAP secondary to systemic vasoconstriction. In the circumflex coronary artery baseline vascular conductance was slightly increased after L-NMMA administration. This small increase can best be explained on the basis of metabolic auregulatory vasodilation secondary to the rise in pressure. Despite the increase in baseline conductance in the circumflex coronary circulation, there was a marked blunting of the dilator responses to acetylcholine (Fig 6). L-NMMA administration did not affect the baseline vascular conductance or the dose-response relationships to ACh in either the mesenteric or femoral vascular beds.

XL-Hgb

Administration of XL-Hgb in addition to L-NMMA caused a large (31% from L-NMMA condition; 39% from baseline) further increase in MAP (Table 2), due again to systemic vasoconstriction with no increases in cardiac output seen. Although there was no change in baseline vascular conductance in the coronary circulation from the L-NMMA condition to the XL-Hgb condition, the change in conductance in response to various doses of ACh (dose-response curve to acetylcholine) was further blunted beyond that noted after administration of L-NMMA. This further blunting beyond that seen with NO-synthase blockade alone, along with the effects of XL-Hgb on arterial pressure, suggest a possible non-NO-mediated vasoconstrictor effect of XL-Hgb. Administration of XL-Hgb caused an increase in the baseline vascular

conductance in both the mesenteric and femoral arteries. However, XL-Hgb had no effect on the dose-response relationships to ACh in either vascular bed. ,

SUMMARY

Changes in baseline vascular tone associated with either albumin or XL-Hgb administration were variable and can be explained by its complex effects on multiple factors including viscosity, vessel tone, oxygen delivery, oxygen demand and metabolic autoregulation. It is difficult to quantify the contribution of each of these factors in each vascular bed studied, and to quantify the importance of changes in each vascular bed to the whole organism. Our observation that XL-Hgb had only mild effects on NO-mediated responses in mesenteric and femoral vascular beds suggests that XL-Hgb might possess other potential vasoconstricting mechanisms that may contribute to the systemic hypertension seen after XL-Hgb administration. Results from Protocol 2 which showed coronary vasoconstriction after XL-Hgb *in excess of* that which occurred after L-NMMA administration lend support to the existence of an extra-endothelial vasoconstricting mechanism.

In summary, XL-Hgb appears to have highly complex effects on regional and systemic circulation. Responses in the coronary vessels were different than those in the femoral and mesenteric arteries. These responses and their potential interactions with various pathophysiologic conditions, should be considered prior to the routine use of hemoglobin-based "blood substitutes" in patients (15).

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Figure 1: Timeline for Protocol 1

The shaded area indicates times of data collection. After instrumentation of the dogs and α- and β-blockade, blood flows were measured in the femoral, mesenteric, and circumflex coronary arteries at rest and during increasing doses of acetylcholine (ACh) and increasing doses of sodium nitroprusside (NTP). Following partial exchange transfusion with either XL-Hgb (N=6) or albumin (N=6), blood flows responses to the same doses of acetylcholine and sodium nitroprusside were repeated.

Figure 2: Timeline for Protocol 2

The shaded area indicated times of data collection. After instrumentation of the dogs, blood flows were measured in the femoral, mesenteric, and circumflex coronary arteries at rest and during increasing doses of acetylcholine (Ach). The blood flow responses to acetylcholine were repeated after administratin of sodium meclofenamate which was used to block any prostaglandin-induced changes in vascular tone. N^G-monomethyl L-arginine (L-NMMA) was given to block nitric oxide synthase activity and acetylcholine dose-responses were repeated. A fourth series of dose-responses to acetylcholine was obtained after a 10% volume load of cross-linked hemoglobin (XL-Hgb).

Figure 3: Baseline Vascular Conductance

Baseline vascular conductance in each arterial bed (femoral, mesenteric, and circumflex coronary) is displayed before and after partial (1/3 blood volume) exchange transfusion with either 5% albumin (N=6) or XL-Hgb (n=6) in an anesthetized canine preparation. After

transfusion with albumin, there was no change in conductance in the femoral artery, and there were increases in the mesenteric and coronary circulations. After transfusion with XL-Hgb, baseline vascular conductance decreased in the femoral artery, was unchanged in the mesenteric circulation, and increased in the coronary artery.

* = $P<0.05$ compared to pre-transfusion value

Figure 4: Dose-Response Curves to Acetylcholine for Protocol 1

The change in conductance from baseline in response to various doses of acetylcholine (ACh) is shown for each arterial bed (femoral, mesenteric, and circumflex coronary) before and after partial (1/3 blood volume) exchange transfusion with either 5% albumin (N=6) or XL-Hgb (n=6) in an anesthetized canine preparation. There was no difference in the femoral artery responses to ACh in either the albumin (4A) or XL-Hgb (4B) conditions. In the mesenteric (4C) and coronary (4E) vascular beds after infusion of albumin, there was an increase in the response to higher doses of ACh. With XL-Hgb, there was no change in the dose-response relationships in the mesenteric vascular bed (4D); however, there was a decrease in the vasodilation in the coronary circulation (4F).

* = $P<0.05$ versus pre-transfusion value

Figure 5: Dose-Response Curves to Sodium Nitroprusside for Protocol 1

The change in conductance from baseline in response to various doses of sodium nitroprusside (NTP) is shown for each arterial bed (femoral, mesenteric, and circumflex coronary) before and after partial (1/3 blood volume) exchange transfusion with either 5%

albumin (N=6) or XL-Hgb (n=6) in an anesthetized canine preparation.There wer no changes in dose-response relationships in the femoral artery after either albumin (5A) or XL-Hgb (5B). There was increased conductance in the mesenteric vascular bed (5C) after albumin, but no change after XL-Hgb (5D). There was an increase in conductance in the coronary artery after albumin (5E) at the lower doses, and a decrease in conductance after XL-Hgb (5F).

* = P<0.05 versus pre-transfusion value

Figure 6: Dose-Response Curves to Acetylcholine for Protocol 2

The change in conductance from baseline in response to various doses of acetylcholine (ACh) is shown for the circumflex coronary artery prior to drug administration, after administration of L-NMMA, and subsequently after 10% volume loading with XL-Hgb in an anesthetized canine preparation (N=5). L-NMMA blunted the vasodilator responses to Ach and administration of XL-Hgb further blunted these vasodilator responses.

* = P<0.05 versus pre-treatment values

+ = P<0.05 versus post L-NMMA values

Table 1: Baseline physiologic values for dogs in Protocol 1. Values are listed before and after partial exchange transfusion with either albumin (N=6) or XL-Hgb (N=6).

Group/Condition	Wt (kg)	Hgb ($gm \cdot dL^{-1}$) Blood/Plasma	MAP (mmHg)	CO ($L \cdot min^{-1}$)	pH	HR ($beats \cdot min^{-1}$)	Temp (°C)
Albumin							
Baseline	17.5±1.2	13.4±0.8 / 0	84±4	2.8±0.3	7.36±0.02	96±4	37.6±0.3
After transfusion	--	7.5±0.7* / 0	76±4*	4.3±0.6*	7.36±0.01	98±6	38.4±0.2
XL-Hgb							
Baseline	17.7±0.4	13.3±0.8* / 0	81±5	2.5±0.2	7.36±0.02	95±6	37.5±0.4
After transfusion	--	9.1±0.7* / 2.6±0.2*	112±8*	2.7±0.4	7.37±0.01	99±5	38.6±0.2

* = $P < 0.05$ versus baseline

Figure 1

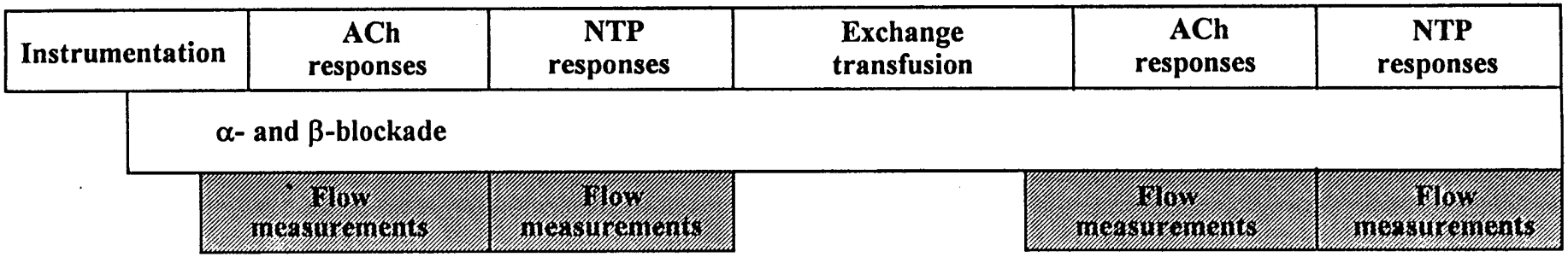

Instrumentation
ACh responses
NTP responses
Exchange transfusion
ACh responses
NTP responses
α- and β-blockade
Flow measurements
Flow measurements
Flow measurements
Flow measurements

Figure 2

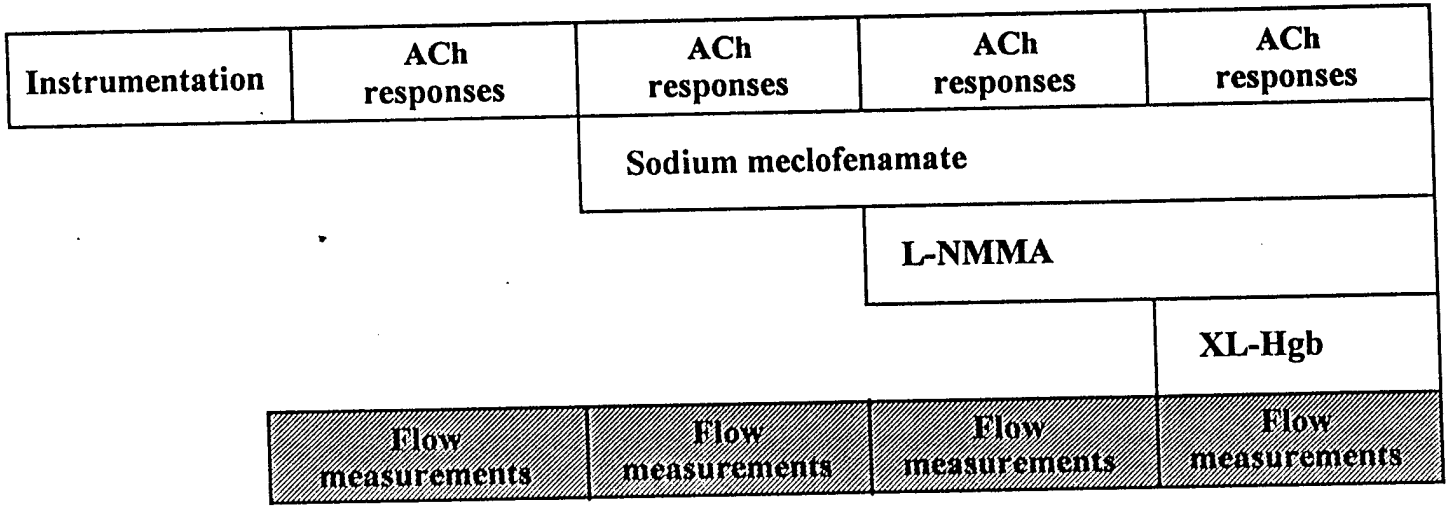
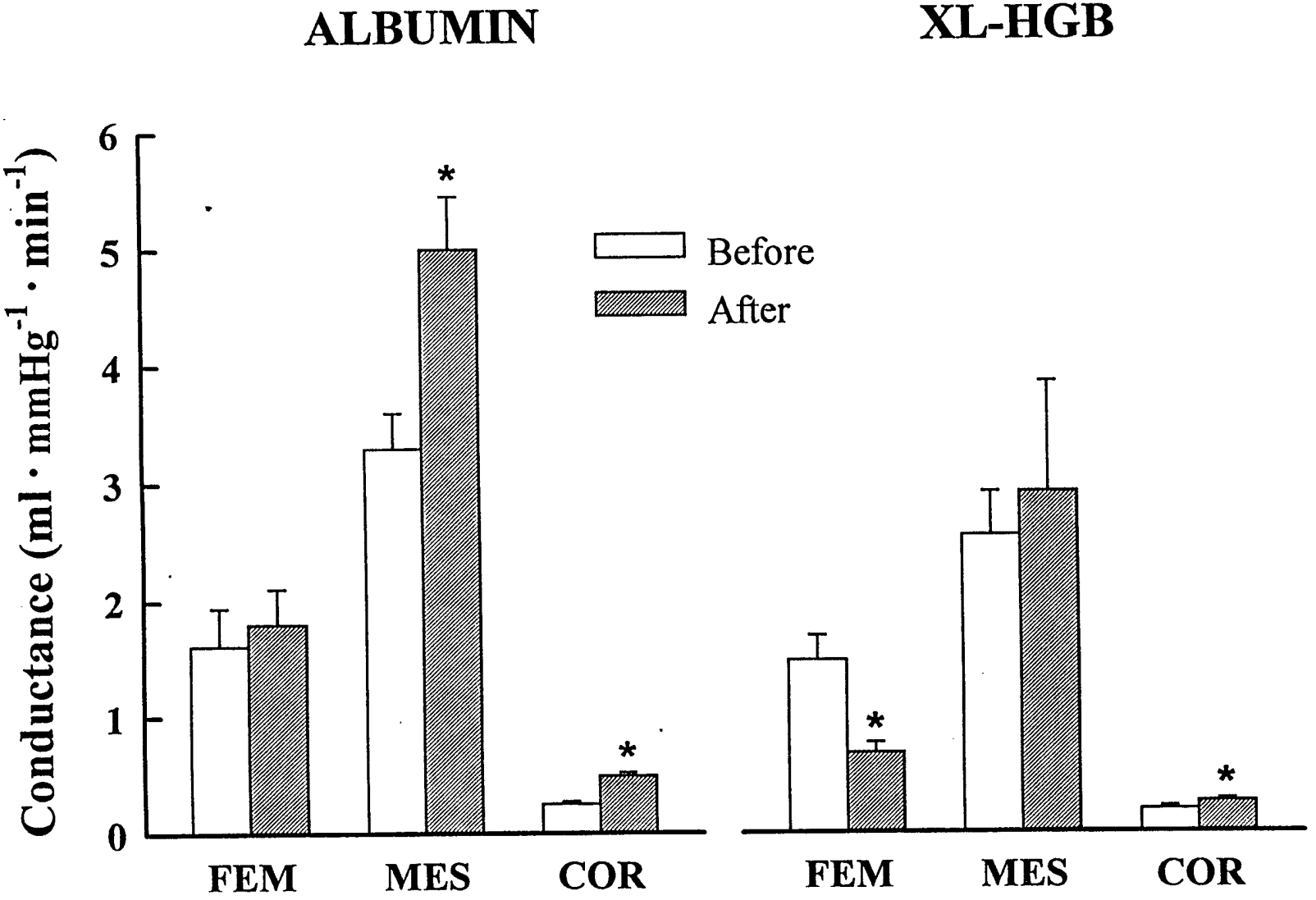

Instrumentation
ACh responses
ACh responses
ACh responses
ACh responses
Sodium meclofenamate
L-NMMA
XL-Hgb
Flow measurements
Flow measurements
Flow measurements
Flow measurements

Figure 3

BASELINE VASCULAR CONDUCTANCE



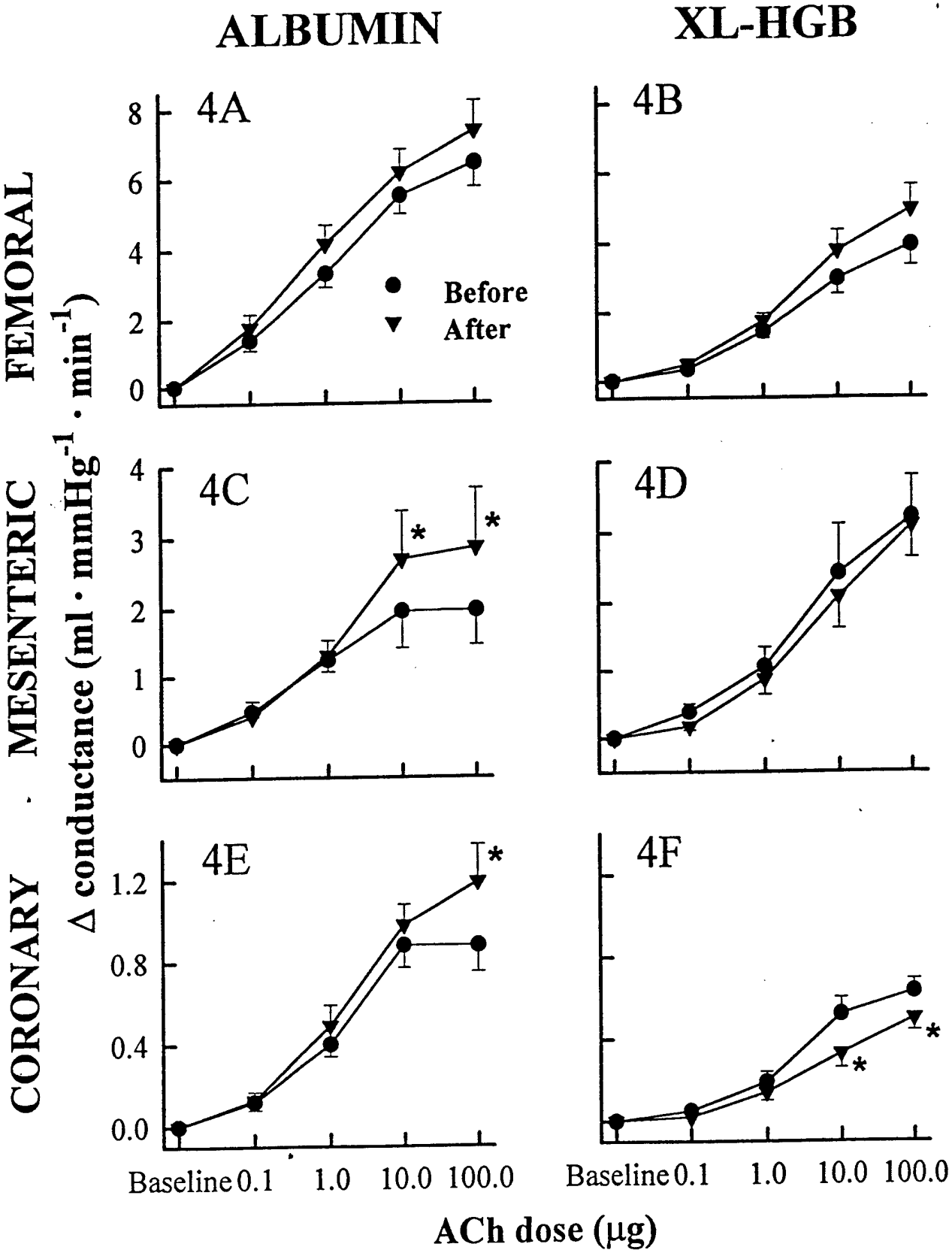
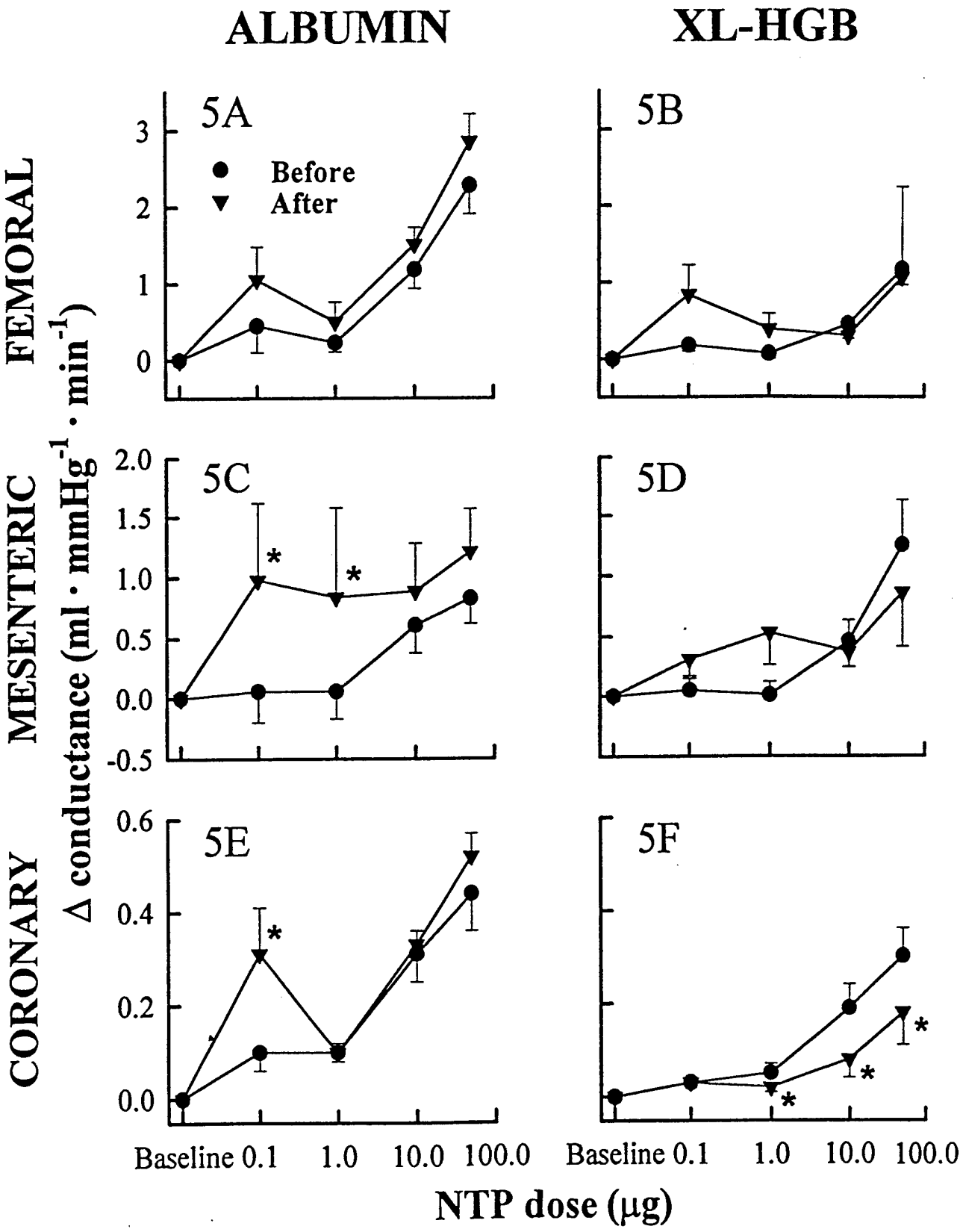

ALBUMIN
XL-HGB
4A
4B
4C
4D
4E
4F
FEMORAL
MESENTERIC
CORONARY
Δ conductance (ml · mmHg⁻¹ · min⁻¹)
Before
After
*
Baseline 0.1 1.0 10.0 100.0
ACh dose (μg)

Figure 4


ALBUMIN
XL-HGB
5A
5B
5C
5D
5E
5F
Before
After
FEMORAL
MESENTERIC
CORONARY
Δ conductance (ml · mmHg^{-1} · min^{-1})
3
2
1
0
2.0
1.5
1.0
0.5
0.0
-0.5
0.6
0.4
0.2
0.0
*
Baseline 0.1 1.0 10.0 100.0
Baseline 0.1 1.0 10.0 100.0
NTP dose (μg)

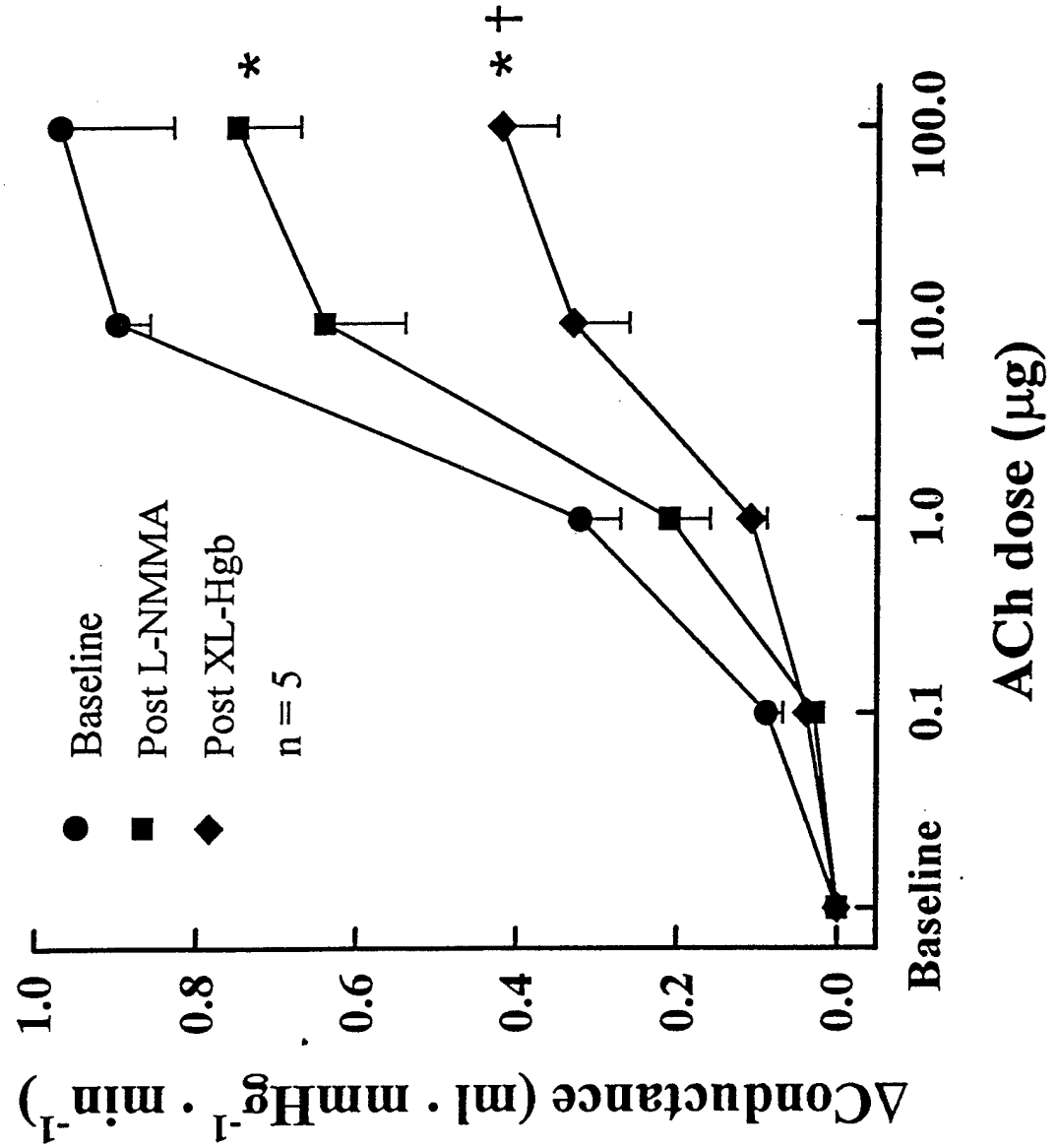

ΔConductance (ml · mmHg^{-1} · min^{-1})
1.0
0.8
0.6
0.4
0.2
0.0
Baseline
Post L-NMMA
Post XL-Hgb
n = 5
*
* +
Baseline
0.1
1.0
10.0
100.0
ACh dose (μg)

Figure 6

APPENDIX

PROJECT 4

"Effects of Xl-Hgb Solution on Renal Blood Pressure Regulating Mechanisms"

Dr. J.C. Romero

#20

Abstract Form

Medical Research

American Heart Association
Fighting Heart Disease and Stroke

P85

50th Annual Fall Conference and Scientific Sessions of the Council for High Blood Pressure Research

September 17-20, 1996
Hyatt Regency Chicago
Chicago, Illinois

Please read carefully.

Deadline date: Abstracts must be received no later than April 15, 1996.

Complete abstract form according to "Rules for Submitting Abstracts."

For review and grading, assign my abstract to the subject area checked below:

- ☐ Cardiac hypertrophy and dysfunction
- ☐ Cell and vascular biology
- ☐ Clinical and experimental hypertension
- ☒ EDRF and other autacoids
- ☐ Molecular biology and genetics
- ☐ Neural regulation of blood pressure
- ☐ Obesity, insulin, and hypertension
- ☐ Receptors and signaling transduction
- ☐ Renal mechanisms and natriuretic factors
- ☐ Vasoactive hormones (renin, kallikrein, etc.)

Heterogenic Vascular Response To Nitric Oxide Synthesis Inhibition

Aleix Cases, J Carlos Romero. Mayo Medical School and Clinic, Rochester, MN.

To test the hypothesis that nitric oxide release plays a role in vascular tone and regional blood flow regulation, we studied the effects of progressive nitric oxide synthesis inhibition on regional and systemic hemodynamics. We infused four progressive doses of L-NAME (.1, 1 ,10 & 50 µg/kg/min) in 45-minute periods in 9 anesthetized dogs. Renal, mesenteric and iliac blood flows (transonic flow probes); aortic pressure (mean arterial pressure); intracavitary pressures and cardiac output (Swan-Ganz) were measured during the experiment. There was a dose-dependent increase in mean systemic and pulmonary arterial pressures noticiable from the second L-NAME dose ($p<0.05$). Cardiac output fell since the initial dose ($p<0.05$), before any change in mean arterial pressure was observed. Systemic and pulmonary vascular resistances increased significantly from the second dose ($p<0.05$). But the percent increase was higher for the pulmonary than for the systemic vascular resistances ($p<0.05$). While iliac blood flow decreased significantly since the first dose ($p<0.05$), mesenteric and renal blood flows decreased only after the third L-NAME dose ($p<0.05$).. The percent decrease of iliac flow was also higher than the renal or mesenteric flows ($p<0.05$). Percent changes in iliac resistances paralleled changes in systemic vascular resistances, except for the last infusion period. These results indicate that nitric oxide synthesis contributes to the regulation of blood pressure and regional vascular tone. Furthermore, the iliac vasculature (skeletal muscle) is more sensitive to nitric oxide synthesis inhibition than the renal and mesenteric vasculature. Importantly, the pulmonary vasculature is also more sensitive to the fall of nitric oxide synthesis than the systemic vasculature.

Check box if applicable

☐ I have submitted the abstract to both the council's Fall Conference and the AHA's annual Scientific Sessions in the following category: _____

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I wish to apply for the ☐ Merck Young Investigator Award; ☐ Merck Young Investigator Award/Latin America; ☐ Hoechst Marion Roussel Hypertension Research Clinical Fellowship Program; ☐ Harry Goldblatt Award in Cardiovascular Research. The appropriate documentation is attached to abstract.

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The author affirms that the material herein will not have been previously published as a manuscript or presented at any national meeting, that any animal studies conform with the "Position of the American Heart Association on Research Animal Use" (*Circulation* 1985;71;849A), and that any human experimentation has been conducted to a protocol approved by the institutional committee on ethics of human investigation or, if no such committee exists, that it conforms with the principles of the Declaration of Helsinki of the World Medical Association (*Clinical Research* 1966; 14:193).

The undersigned certifies that all authors named in the abstract have agreed to its submission for presentation at the annual Fall Conference and Scientific Sessions of the Council for High Blood Pressure Research or the AHA's annual Scientific Sessions and are aware of the rule cited above that may disqualify it for consideration for the AHA's annual Scientific Sessions.

Author's signature _____

HEMODYNAMIC AND RENAL EFFECTS OF CROSS-LINKED HEMOGLOBIN INFUSION

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Running Title: Cross-linked hemoglobin administration in dogs

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ABSTRACT

It is well known that hemoglobin binds nitric oxide producing a pronounced vasoconstriction in isolated arteries. However, it is debatable whether or not such an effect takes place in whole animals, because hemoglobin also is known to catalyze the formation of prostaglandins from arachidonic acid. Acute studies were performed to evaluate the effects induced by intravenous infusion of cross-linked hemoglobin (XL-Hb) on blood pressure and renal, iliac, and mesenteric flows, as well as on renal function in 6 anesthetized dogs. A similar volume-matched expansion with 6% Dextran was used as control (n=6). Glomerular filtration rate (GFR), urinary flow, and total and fractional sodium excretion were measured before and after XL-Hb or dextran infusion to evaluate possible renal function changes. XL-Hb administration resulted in a 29 % elevation in BP and a significant decrease of blood flow (30-37%) to the three vascular beds. XL-Hb did not alter GFR or sodium excretion, despite the increase in BP. In contrast, the administration of Dextran did not significantly alter BP but induced a significant increase (6-13%) of blood flow in the three vascular beds. These changes were accompanied by three-fold increases in urinary flow and sodium excretion without alterations in GFR. The binding effect of XL-Hb on NO was studied in isolated renal arteries in organ chambers. These *in vitro* studies demonstrated that XL-Hb blunted the endothelium-mediated vasodilator response to the calcium ionophore A23187 and to acetylcholine. Our results demonstrate that XL-Hb administration is followed by hypertension, vasoconstriction and blunted natriuresis. All these effects are compatible with the scavenging effect on NO attributed to XL-Hb.

Index Terms: Dextran, nitric oxide, prostaglandins

INTRODUCTION

It is well known that the paramagnetic properties (odd number of electrons) of nitric oxide (NO) account for a remarkable binding affinity for the heme iron complex (8). Such characteristic accounts for both the NO-activation of guanylate cyclase as NO binds the heme group of this enzyme, and the inactivation of NO by hemoglobin (Hb) (1). This later effect has been well described in isolated arteries, but it has never been explored in whole animals (9,10). From a speculative point of view, a significant uptake of NO in systemic circulation may lead to a vasoconstriction if the binding to Hb imposed a reduction on the amount of endothelial NO which diffuses towards the vascular smooth muscle. However, there are also experimental evidences showing that Hb catalyzes the transformation of arachidonic acid to prostaglandins with remarkable specificity (3,4,16,22). Such a cyclooxygenase-like activity could stimulate the formation of vasodilators, such as PGI_2 or PGE_2 (25-26), which may decrease systemic blood pressure. This effect would counteract the vasopressor action of NO suppression.

Until recently, the possibility of testing the validity of these assumptions was precluded by the instability and rapid breakdown of stroma-free hemoglobin. Such a problem has been recently overcome by the synthesis of different forms of cross-linked hemoglobin (XL-Hb). One of these compounds is hemoglobin cross-link alpha-alpha with bis (3,5-dibromosalicyl) fumarate (7). This chemical modification increases the half-life of Hb in circulation and reduces its renal clearance, thus prolonging intravascular retention (7). As it is apparent, the potential clinical use of this compound as a blood substitute (8,24) creates an additional interest in studying the hemodynamic effects of free hemoglobin in circulation.

It should be mentioned here that Shultz et al (20) showed that the intravenous of administration of diaspirin cross-linked hemoglobin to Sprague Dowley rats produced a transient increase of blood pressure. However, no attempts were made in this study to determine if a vasoconstrictor effect of cross-linked hemoglobin were uniformly exerted in different vascular beds or which were the specific changes produce by cross-linked hemoglobin in renal function and urine sodium excretion. Such information on extracellular fluid volume homeostasis is very critical when

evaluating the characteristics of a volume expander such as cross-linked hemoglobin.

This study was therefore undertaken to define the hemodynamic changes induced by the intravenous infusion of XL-Hb on three vascular beds: iliac, mesenteric, and renal. These vascular beds were selected as they are important contributors of total peripheral resistance (5) and their diversity in metabolic activities justify exploring different responses depending on NO and/or PG's involvement. In these studies, the concomitant changes in blood pressure and renal excretory function, namely glomerular filtration rate and urinary sodium excretion, were also monitored. The results of these studies were compared to the hemodynamic effects produced by equiosmolar concentrations of dextran. This substance was chosen over whole blood, plasma or albumin because its molecular weight is comparable to that of XL-Hb and it is biologically neutral. This characteristic help to distinguish the hemodynamic effects that could be derived from the XL-Hb-induced volume expansion, exempted from its biological effects.

To determine if XL-Hb produces the same vasoconstriction than that attributed to the NO scavenging actions of hemoglobin, we characterized the effects of XL-Hb on the relaxation induced by either calcium ionophore A23187 or acetylcholine in isolated renal arteries, which are maneuvers that stimulate the synthesis of NO.

MATERIALS AND METHODS

Intravenous infusion of XL-Hb or dextran

Twelve male mongrel dogs (15-20 kg) were anesthetized with 30 mg/kg of intravenous sodium pentobarbital and ventilated according to the nomogram of Kleiman and Radford (13). The femoral artery was catheterized for continuous blood pressure monitoring and to collect blood samples; while the femoral vein was cannulated for infusion of creatinine (20 mg/min) to measure GFR, and additional anesthesia, as well as to infuse XL-Hb or dextran. Through a left flank incision transonic flow probes (Transonic Systems, Inc., New York, USA) were placed in the mesenteric, renal and iliac segments proximal to the aorta for continuous blood flow monitoring. A curved 23-

gauge needle was inserted into each of these arteries at the distal segment to avoid interferences with flow measurement. The needles were connected via PE 50 tubing to injection ports attached to syringe pumps. Saline was continuously infused, 0.5 ml/min, into each vascular bed. Bolus injections of two doses of arachidonic acid (AA) (205 nM and 410 nM in the iliac and 410 nM and 820 nM in the renal and mesenteric arteries) were injected into each vascular bed before and one hour after volume expansion to detect possible changes in vascular reactivity due to enhanced prostaglandin formation produced by the catalytic actions of XL-Hb. The left ureter was also cannulated to collect urine samples.

Before XL-Hb or dextran infusions were started, averaged values from two 20 min. periods were considered for basal situation (periods 1 and 2). The infusion of either 6% Dextran or XL-Hb (10% blood volume) was given by continuous infusion over 20 minutes. Thereafter, three 20 min. periods (3,4, and 5) were considered to evaluate the effects of the two substances. Urine samples were collected during each clearance period to measure urine flow, total and fractional Na^+ excretion rates, osmolality and creatinine levels. Blood samples for measuring plasma creatinine and hematocrit levels were collected at the midpoint of each clearance period, while samples to measure plasma renin activity (PRA) and atrial natriuretic peptide (ANP) were obtained at the end of the first control period and 40 minutes after the infusion (end of period 4).

Plasma and urine creatinine were measured using a Beckman Creatinine Analyzer, and creatinine clearance was used to estimate GFR. Osmolality was measured by a freezing point depression osmometer (Precision System 5004); Na^+ concentration was measured using a flame photometer (Instrumentation Laboratory IL943). Finally PRA and ANP were measured by commercial radioimmunoassay Kits (DuPont NEA-105 and Peninsula RIK-8798, respectively).

In vitro effects of XL-Hb in isolated renal arteries

The experiments were performed on rings (3-5 mm in length) of renal arteries taken from dogs (15-20 kg) anesthetized with sodium pentobarbital (30 mg/kg iv) and euthanized via exanguination. The arteries were placed in modified Krebs-Ringer bicarbonate solution [control solution (in mM): 118.3 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1.2 $MgSO_4$, 1.2 KH_2PO_4, 25.0 $NaHCO_3$, 0.026 calcium EDTA,

and 11.1 glucose]. Each ring was connected to an isometric force transducer (Gould UTC-2, Oxnard, CA, USA) and suspended in an organ chamber filled with 25 ml of control solution (37°C, pH 7.4) and gassed with 94% O_2-6% CO_2 . Isometric tension was recorded continuously.

Each ring was gradually stretched to the optimal point of its length-tension curve as determined by the contractions to norepinephrine (3×10^{-7} M) (13). Optimal resting tensions were 10 g for renal arteries (12). The functional integrity of endothelium was tested by the presence of relaxations to acetylcholine (10^{-6}M).

The following pharmacological agents were used: acetylcholine hydrochloride (Sigma, St. Louis, MO), calcium ionophore A23187 (Sigma), L-norepinephrine (Sigma) and papaverine hydrochloride (Sigma). Stock solutions of the drugs were prepared fresh every day. Drugs were dissolved in distilled water such that volumes of <0.2 ml were added to the organ chambers. All concentrations are expressed as final molar (M) concentration in the bath solution.

Cross-linked hemoglobin was obtained from Walter Reed Army Institute of Research (Washington D.C., USA). The solution was prepared from stroma-free human hemoglobin from outdated blood modified with bis (3,5-dibromosalicyl) fumarate according to the method of Snyder (21). The cross-linked hemoglobin was formulated in Ringer acetate (7 g/100 ml) and maintained at 4°C until the day of use. At that time it was passed through a 0.22 μm filter to remove particulate matter, then warmed to 37°C by placing the bag in a water bath. The incubation time for XL-Hb was 30 min.

Concentration-response curves were obtained in a cumulative fashion. Several rings cut from the same artery were studied in parallel; only one concentration-response curve was made per preparation. The relaxations were expressed as a percentage of maximal relaxations to papaverine (3×10^{-4}M).

Statistical analysis.

The results are expressed as means ± SEM. Results from the two control periods were averaged and compared to each of the post infusion periods with a randomized block analysis of variance. When the F value yielded a $p<0.05$, difference between clearances were determined by

Newman-Keuls multiple range test. Differences between Dextran and XL-Hb infusions were evaluated using an unpaired Student's t-test. With respect to the *in vitro* studies, n refers to the number of dogs and the statistical evaluation of the data was performed by Student's t-test for paired observations. A $p<0.05$ was considered significant.

RESULTS

Infusion of XL-Hb

Infusion of XL-Hb induced a 13.5% decrease in hematocrit levels (from 39.5±2.06% to 34.17±1.23%, $p<0.01$). This infusion (control value of periods 1 and 2 vs. averaged increments in periods 3-5) produced significant and sustained decreases in mesenteric (210±27 to 147±22 ml/min, $p<0.05$), renal (198±14 to 134±12 ml/min, $p<0.05$), and iliac (135±17 to 82±10 ml/min, $p<0.05$) blood flows (Fig. 1b-d) while mean arterial pressure increased significantly from 114±4 to 147±10 mm Hg ($p<0.05$) (Fig. 1a). GFR (Fig. 2a) remained unchanged, as well as total urinary and fractional sodium excretion (Fig. 2b-c). Urinary flow during XL-Hb administration increased by 81±42.4%. In addition, XL-Hb administration resulted in a 75% decrease in plasma renin activity and a 168% increase in atrial natriuretic peptide levels (Table 1).

Finally, in the three vascular beds the two doses of AA systematically increased blood flow after the infusion of XL-Hb (Fig. 3a-c), but not in the basal period.

Infusion of Dextran

Dextran infusion induced a decrease of 13.8 % of hematocrit levels (from 36.17 ± 1.5% to 31.17 ± 1.67%, $p< 0.01$). In contrast with the effects of XL-Hb, dextran infusion produced significant increases in mesenteric (346±43 to 391±34 ml/min, $p<0.05$), renal (182±23 to 211 ±27 ml/min, $p<0.05$), and a transient increase in iliac (176±23 to 186±17 ml/min) blood flows (Fig. 1b-d), without concomitant changes in mean arterial blood pressure (Fig. 1a). Sodium excretion and fractional sodium excretion (Fig. 2b-c) rates significantly increased from 51±17 to 168±44 μEq/min,

($p<0.05$) ml/min ($p<0.05$) and from 0.96±.32 to 2.78±0.96% ($p<0.05$), respectively, without any change in GFR (Fig. 2a). The lack of changes in GFR associated to the significant increments in sodium excretion resulted in a significant elevation of the calculated FeNa which were comparable to the increments seen for total sodium excretion (2c). Urinary flow increased by 206.8±43.9% ($p=0.066$ with respect to the increase observed in XL-Hb group). In addition, Dextran infusion resulted in a 47% decrease in plasma renin activity and a 16% increase in atrial natriuretic peptide levels (Table 1).

Intraarterial bolus injections of AA (Fig. 3a-c) did not alter blood flow in any vascular bed before or after Dextran infusion.

Effects of XL-Hb on renal artery relaxation *in vitro* induced by A23187 and acetylcholine

It can be seen in Figure 4 that under control conditions exposure of renal arteries to concentrations of A23187 of 8, 7.5, and 7 (-logM) evoke a relaxation of 20%, 66%, and 95%, respectively. The vasodilator effect was significantly blunted by the administration of XL-Hb (10^{-6} M), since the administration of the first two doses of A23187 (8 and 7.5, -logM) failed to produce a change in the basal tone, whereas the concentration of -7 logM evoked only a 50% relaxation of the arterial strips. This represents a 50% decrease with respect to the relaxation evoked by the same dose of A23187 in the absence of XL-Hb.

DISCUSSION

Since Dextran (MW 55,300) and XL-Hb (MW 64,000) have high molecular weights and both solutions were matched for osmolality and sodium content, it would be reasonable to assume that the magnitude of both volume infusions was comparable. In fact, the average fall of hematocrit in both

groups of dogs, 13.8% and 13.5% respectively, was similar. In spite of these similarities, the consequences derived from the intravenous infusion of both substances were markedly different. The acute infusion of XL-Hb was followed by an increase in MAP which was accompanied by peripheral vasoconstriction in several vascular beds. In fact, the estimated blood flows in renal, mesenteric, and iliac vasculatures were uniformly decreased by 32, 30, and 39%, respectively. The increase in intrarenal resistance seen during the XL-Hb infusion was equally distributed between glomerular afferent and efferent vasculature as GFR did not change. Under these conditions, urinary volume, and total and fractional excretion of sodium remained within the range of values recorded in the control periods, despite the volume expansion induced by the infusion and the increase in blood pressure. This fact indicates that the increase in systemic blood pressure due to the administration of XL-Hb failed to produce pressure-induced natriuresis. An important issue disclosed by our results shows that the hemodynamic and renal effects produced by XL-Hb differs from those produced by a neutral volume expander of approximately the same molecular weight, such as dextran.

It has been previously reported that the intravenous administration of Dextran produces a significant increase in cardiac output which fails to increase mean arterial pressure because of a compensatory reduction in total peripheral resistance (2). These results are in agreement with our findings which show that Dextran infusion did not modify blood pressure levels, but increased transiently the iliac and sustainedly the mesenteric and renal blood flows.

The increase in RBF produced by Dextran was not accompanied by changes in GFR, which suggests that the renal vasodilatation affected similarly both glomerular afferent and efferent arterioles in such a manner that glomerular capillary pressure remained fairly constant. However, urine flow and total and fractional Na^+ excretion were significantly increased, thus indicating that the major cause for the observed natriuresis consisted of a reduction of tubular sodium reabsorption (23). A decrease in tubular reabsortion under these conditions has been attributed to changes in glomerular-tubular balance, to a decrease in PRA, as well as to a withdrawal of the renal sympathetic activity (2,23). Furthermore, there is numerous evidence pointing out that the volume expansion-induced natriuresis is very significantly mediated by the elevation of ANP (6) and by the stimulation of NO

synthesis (17). Our results are also in agreement with some of these previous observations since Dextran infusion was attended by a significant fall in PRA and by a marked elevation in the circulating levels of ANP.

It has been reported that XL-Hb exerts an effective scavenging action on circulating NO, as this molecule possesses a high affinity for the heme groups (9,10). The scavenging of NO could account for the rise in blood pressure and the decrease in the three regional blood flows, as well as the blunted natriuresis, that we found in our study. This statement is supported by comparable results which were observed when L-NAME, a potent inhibitor of NO synthesis, was infused into rats (14) and dogs (18-19). The response in these animals involves the elevation of MAP without a proportional increase in sodium excretion because of the counteracting antinatriuretic effect of NO suppression (17). Additional support to the idea that the vasoconstrictor effect of XL-Hb seen in our *in vivo* study is due to the scavenging effect of NO is provided by our observations *in vitro* in isolated renal arteries. This experiment shows that HL-Hb blunted the vasodilatory response induced by two known endothelium-dependent vasodilators, such as acetylcholine and A23187.

Furthermore, in a previous study conducted by Schultz, et al (20) it was shown that the administration of diaspirin cross-linked hemoglobin (DCL-Hb) produced a significant increase of MAP which after reaching the peak was significantly reduced by the intravenous infusion of NO donors (such as nitroglycerine, NTG) or NO synthesis precursors (such as L-arginine). In the absence of appropriate controls these results are difficult to interpret because the vasodilator effect of NO donors could reduce any kind of hypertension. On the other hand, the hypotensive effect of L-argenine may be indicating that this amino acid is capable of increasing the producion of NO to a point that overrides the scavenging effect of DCL-Hb or that the vasoconstrictor effects of DCL-Hb are not due to the binding of NO by the HEM group. The authors favored the first possibility because they show that the inactivation of the HEM group by conversion to cyanomethemoglobin fail to induce hypertension. The concept that NO in any form would react with oxygenated HEM groups inactivating the NO and leaving a positive charge on the molecules of hemoglobin is at present highly elevated. Jia, et al (11) have recently shown that NO would preferentially react with a thiol (a sulfur

and hydrogen) -group of the two cisteine molecules contained in hemoglobin; while the binding of NO to the HEM group has a lower affinity.

The biological activity of hemoglobin containing NO bound to the thiol groups only; or to the HEM groups only or to both groups was tested by Jia, et al (11) in isolated arteries. It was found that the vessels constricted to all three hemoglobin preparations but the constrictor effect was greater when both the thiol and HEM groups did not contain NO. From our results we cannot determine which chemical group was responsible for binding NO. However, it is conceivable that the continuous uptake of NO by XL-Hb from the lumen of the vessel may create a low concentration gradient of NO which will decrease the diffusion of NO toward the smooth muscle.

The cyclooxygenase-like activity of the heme group has been well characterized *in vitro* (3,4,16,22, 25-26). However, the hypertensive effect that we have achieved during XL-Hb administration does not seem to agree with these *in vitro* findings. In fact, our results support the idea that Hb-dependent stimulation of PG synthesis may be of a rare occurrence under physiological conditions when all hemoglobin is contained in the red cells or even circulating free into the vascular compartment as it was the case of XL-Hb (25). In fact, this cyclooxygenase-like effect of Hb was apparent only after an intravenous bolus infusion of the substrate was given. Furthermore, the predominant effect produced by the infusion of XL-Hb was a generalized vasoconstriction in all vascular beds studied.

An interesting and novel finding of our study was the pronounced elevation of circulating ANP observed during the infusion of XL-Hb. This increase cannot be ascribed to a volume expansion as it was 11-fold higher than the increase induced by a similar volume expansion induced by the infusion of Dextran. Although our study does not allow further speculation of the mechanism by which XL-Hb influenced the concentration of ANP in blood, there are evidences showing that ANP release could be stimulated by changes in the production of humoral factors derived from the endothelial cells (15). In our study, the 2.7-fold higher increase of circulating ANP achieved with XL-Hb expansion (compared to Dextran) was not associated with a proportional increase in Na^+ excretion. This fact suggests that XL-Hb produced a blunted natriuresis despite the higher increase in

ANP levels observed in the XL-Hb group.

In summary, this study demonstrates that the acute infusion of XL-Hb into euvolemic dogs induces a significant vasoconstrictor effect in three major vascular beds (renal, mesenteric, and iliac), leading to an increase in blood pressure, and a blunted natriuresis. These alterations can not be attributed to volume expansion, as they were not observed when a similar expansion was induced with Dextran. Therefore, these differences in the responses may be more related to specific biological actions of XL-Hb, such as an NO scavenging effect. This statement is supported by the fact that NO synthesis inhibition with L-NAME induces comparable effects to those obtained with XL-Hb, as well as the effect of XL-Hb in isolated renal arteries in the present study. Finally, the possible stimulation of vasodilator prostaglandin synthesis during XL-Hb infusion was not observed in our *in vivo* studies, as indicated by the elevation of blood pressure and the reduction in the three arterial blood flows.

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FIGURE LEGENDS

Figure 1a-d. Changes in mean arterial pressure (MAP) and in iliac (IBF), mesenteric (MBF), and renal (RBF) blood flows observed after the i.v. infusion of Dextran (closed circles) or XL-Hb (open circles) during periods 2, 3, 4, and 5. Period 1 served as a baseline.

* $p<0.05$ between the treatment groups and † $p<0.05$ with respect to the basal period

Figure 2a-c. Changes in glomerular filtration rate (GFR), urinary sodium excretion (UNaV) and fractional excretion of sodium (FeNa) during the same conditions explained in the previous figure.

* $p<0.05$ between the treatment groups and † $p<0.05$ with respect to the basal period

Figure 3a-d. Percent (%) increase in iliac (IBF), mesenteric (MBF) and renal (RBF) blood flows induced by the bolus injection of two doses of arachidonic acid (AA) given during the control periods and after infusion of Dextran or XL-Hb (see reference bars).

* $p<0.05$ with respect to basal period

Figure 4a-b. Concentration-response curves to A23187 in canine renal arteries in the absence and presence of XL-Hb. Relaxations were obtained during contractions to norepinephrine (3×10^{-7} M). Data are shown as means±SE and expressed as percent of maximal relaxation induced by papaverine (3×10^{-4} M, n=5 for control rings and in the presence of XL-Hb, respectively).

* $p<0.05$ with respect to control rings.

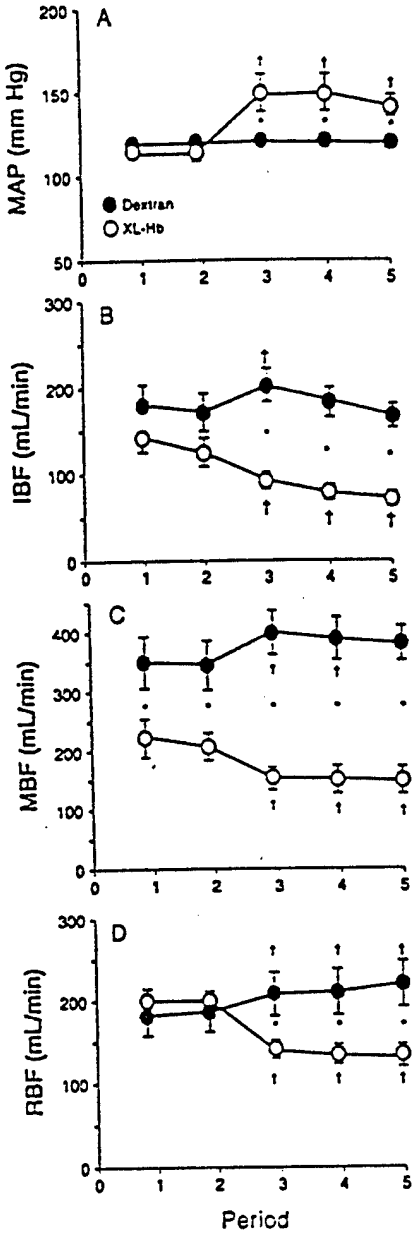

Figure 1a-d: Changes in mean arterial pressure (MAP) and in iliac (IBF), mesenteric (MBF), and renal (RBF) blood flows observed after the intravenous infusion of Dextran (closed circles) or cross-linked hemoglobin (XL-Hb) (open circles) during periods 2,3,4, and 5. Period 1 served as a baseline.

*$p<.05$ between the treatment groups

+$p<.05$ with respect to the basal period.

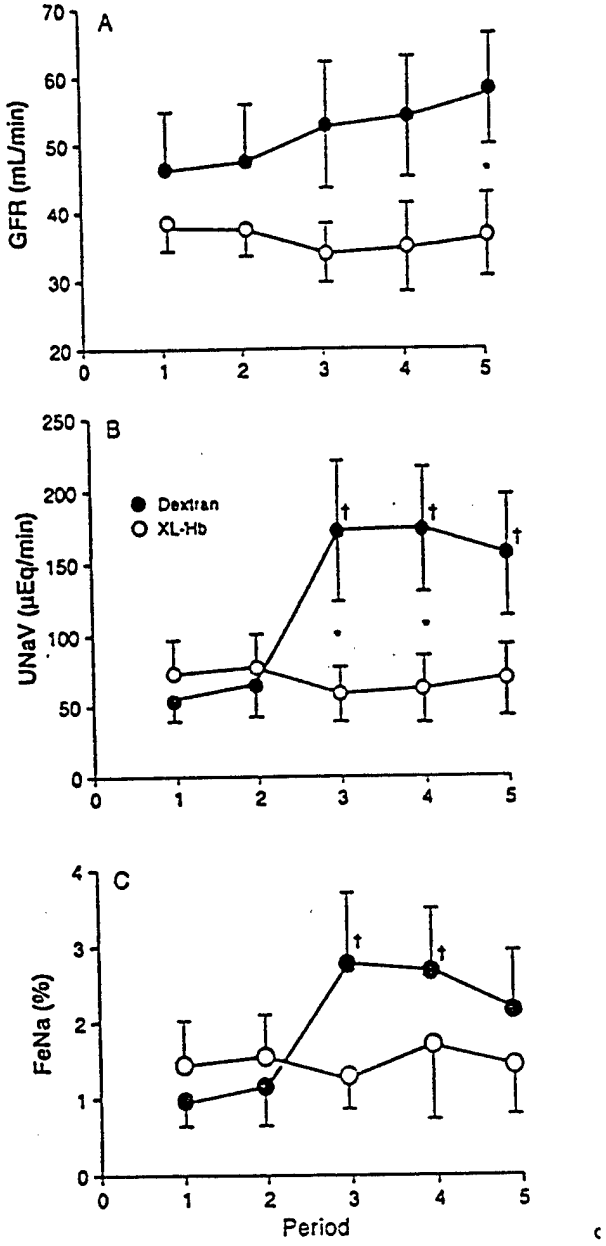

Figure 2a-c. Changes in glomerular filtration rate (GFR), urinary sodium excretion (UNaV) and fractional sodium excretion (FeNA) during the same conditions explained in the previous figure.

* $p<.05$ between the treatment groups

+ $p<.05$ with respect to the basal period.

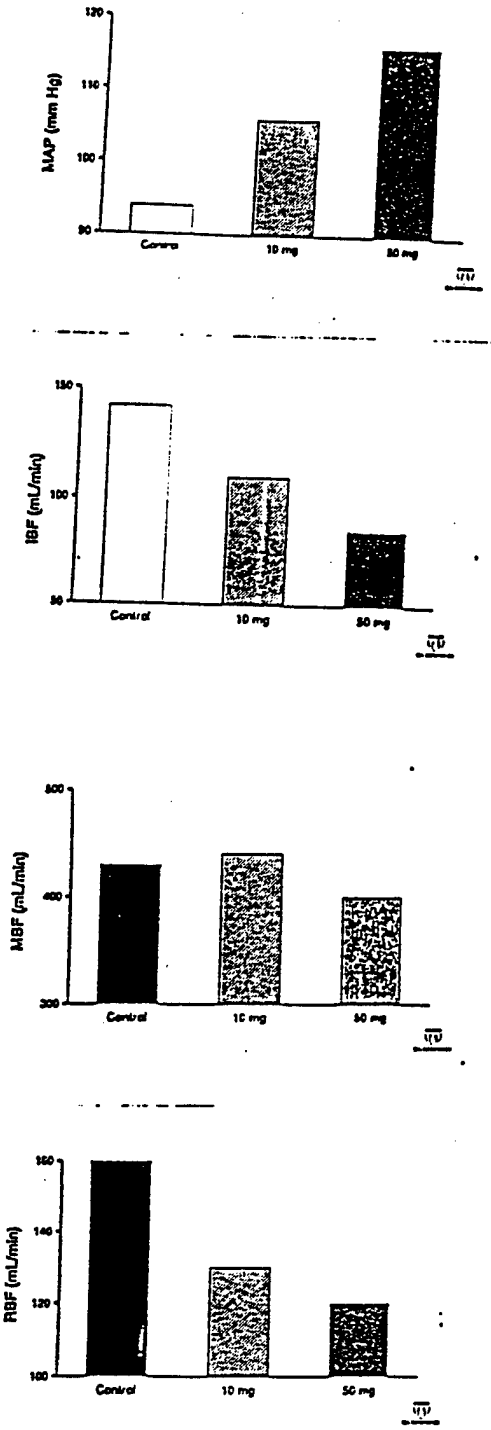

Figure 3 a-d. Response of mean arterial pressure (MAP) and in iliac (IBF), mesenteric (MBF), and renal (RBF) blood flows to the I.V. infusion of 10 and 50 mg/kg body weight (b.w.) of L-NAME in nine dogs.

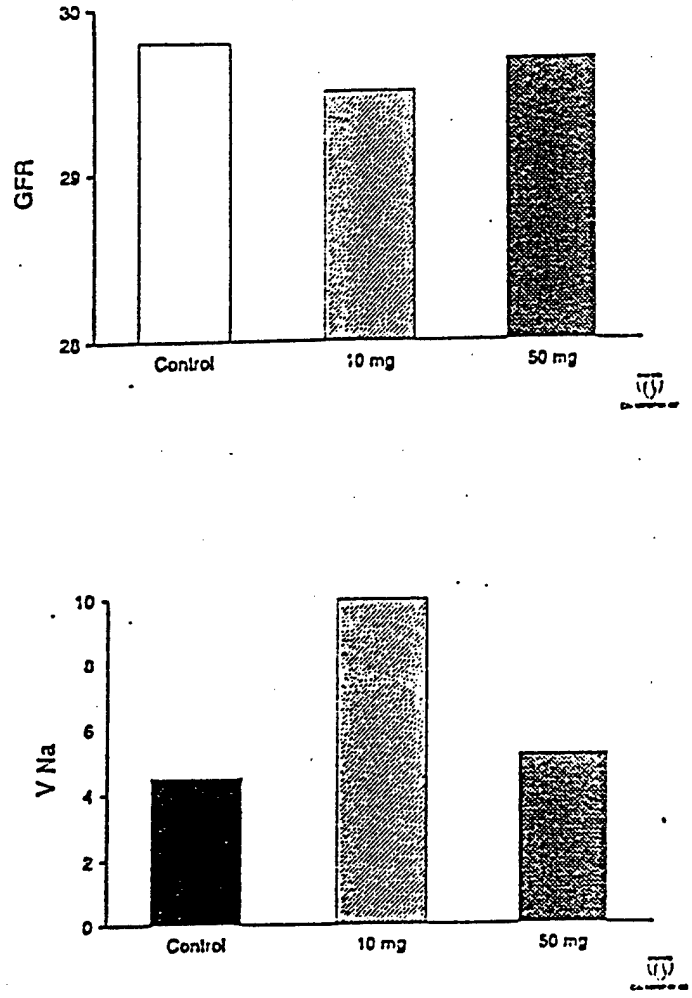

Figure 4 a-b. Response of glomerular filtration rate (GFR) and urinary sodium excretion (UNa) to the I.V. infusion of 10 and 50 mg/kg b.w. of L-NAME.

Changes in mean arterial pressure, blood flow to the three vascular beds, glomerular filtration rate, and urinary sodium excretion in response to the highest dose of L-NAME, paralleled the changes seen with the intravenous infusion of cross-linked hemoglobin. These findings support the notion that the biological actions of cross-linked hemoglobin may be related to its scavenging of nitric oxide.

Table 1. Hormonal values obtained before and after infusion of dextran or XL-Hb

	Dextran Control	Dextran Infusion	XL-Hb Control	XL-Hb Infusion
PRA (ng AI/ml/hr)	3.4±1.6	1.8 ±0.5	5.6 ±0.8	1.4 ±0.6*
ANP (pg/ml)	72 ± 9	83 ±12*	103 ± 13	276±34*

Mean ±SEM. * $p<0.05$ vs. basal period. PRA: plasma renin activity. ANP: atrial natriuretic peptide.

PERSPECTIVES

The therapeutic efficacy of blood transfusion has been hampered by the existence of transmissible disease such as AIDS and by accidents linked to blood storage. These problems could now be solved by using stroma-free solutions of newly polymerized hemoglobin (this component has been cross-linked hemoglobin) XL-Hb. The study shows that the I.V. infusion of XL-Hb differs from the effect produced by other volume expanders, such as Dextran, because it induces a marked increase in peripheral vascular resistance (such as renal mesenteric and iliac vasculatures) with a marked elevation of mean arterial pressure. These changes, however, are not accompanied by any alteration in sodium excretion. This hypertensive an anti-natriuretic effects are most likely produced by a reduction in the concentration of NO which is bound to hemoglobin. These actions will have to be taken in consideration if cross-linked hemoglobin is used as a volume expander in hypovolemic conditions in humans.